CORD BLOOD LEPTIN AND C-PEPTIDE IN INFANTS BORN TO DIABETIC MOTHERS

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BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled **CORD BLOOD LEPTIN AND C-PEPTIDE IN INFANTS BORN TO DIABETIC MOTHERS** is the original bonafide work done by **Dr. K VANI**, post graduate student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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LIST OF ABBREVIATIONS

1. DM – Diabetes Mellitus
2. GDM – Gestational Diabetes Mellitus
3. FPG – Fasting Plasma glucose
4. PPG – Post Prandial Plasma glucose
5. LDL-c – Low density lipoprotein-cholesterol
6. VLDL-c – Very Low density lipoprotein-cholesterol
7. FFA – Free fatty acid
8. OGTT – Oral Glucose Tolerance Test
9. ADA – American Diabetes Association
10. TGL – Triglycerides
11. TNF-α – Tumor necrosis factor – α
12. IP3 – Inositol Triphosphate
13. IGF – Insulin- like Growth factor- 1
14. IRS – Insulin Receptor Substrate
15. BMI – Body Mass Index
16. IL-8 – Interleukin 8
17. GLUT – Glucose Transporter
18. MSH – Melanocyte Stimulating Hormone
19. NPY – Neuropeptide Y
20. AgRP – Agouti-related peptide
21. MAPK – Mitogenic Activated Protein Kinase
22. WAT – White Adipose Tissue
23. BAT – Brown Adipose Tissue

INTRODUCTION

INTRODUCTION

Diabetes during pregnancy is associated with increase in maternal and perinatal morbidity. The hallmark of this condition is increased insulin resistance. Maternal hormones are thought to interfere with the action of insulin as it binds to the insulin receptor. Since insulin promotes the entry of glucose into most cells, insulin resistance prevents glucose from entering the cells. As a result, glucose remains in the bloodstream, where glucose levels rise. More insulin is needed to overcome this resistance; more insulin is produced than in a normal pregnancy. Macrosomia[1], congenital cardiac and central nervous system anomalies, skeletal malformations and respiratory distress syndrome are some of the well known complications occurring in infants of diabetic mothers.

Moreover, human epidemiological and animal studies suggest that the intrauterine diabetic environment increases the risk of hypertension, obesity, and type II diabetes in adulthood in the offspring of diabetic mothers. Fetal hyperinsulinemia at birth acts as a marker of this risk and it may also have potential prognostic implications. Thus, higher insulin levels in utero might be a cause of later metabolic complications [5,6,7].

Leptin is a hormone of adipose tissue that plays a key role in regulating energy intake and expenditure and the level of circulating leptin is directly proportional to the total amount of fat in the body. Leptin also has direct metabolic effects on several tissues in promoting lipolysis. Cord blood leptin correlates with measures of adiposity at birth[2,3,4] and is also raised in offspring of mothers with diabetes [3,4].

The interactions between leptin and insulin could play a very important role in the pathogenesis of GDM and type 2 DM in pregnancy. So the assessment of

insulin and leptin at birth may be a particularly useful way of monitoring whether the fetus has been exposed to abnormally high levels of glucose in utero.

We have measured the concentrations of C-peptide and leptin in cord blood together with birthweight and other anthropometric measures at birth. The correlation between leptin, C-peptide and the anthropometric measures like birthweight, ponderal index and head circumference, is also assessed in this study.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

DIABETES MELLITUS

Diabetes mellitus (DM) refers to a group of common metabolic disorders that share the phenotype of hyperglycemia. Several distinct types of DM exist and are caused by a complex interaction of genetics and environmental factors. Depending on the etiology of the DM, factors contributing to hyperglycemia include reduced insulin secretion, decreased glucose utilization and increased glucose production. With an increasing incidence worldwide, DM will be a leading cause of morbidity and mortality for the foreseeable future.

CLASSIFICATION

DM is classified on the basis of the pathogenic process that leads to hyperglycemia. The two broad categories of DM are designated type 1 and type 2.

- Type 1 diabetes is the result of complete or near-total insulin deficiency. It is characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas leading to insulin deficiency. This type of diabetes can be further classified as immune-mediated or idiopathic. The majority of type 1 diabetes is of the immune-mediated nature, where beta cell loss is a T-cell mediated autoimmune attack.

- Type 2 DM is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion and increased glucose production. Distinct genetic and metabolic defects in insulin action and/or secretion give rise to the common phenotype of hyperglycemia in type 2 DM. Type 2 DM is preceded by a period of abnormal glucose homeostasis classified as impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). The disease is polygenic and multifactorial since in addition to genetic susceptibility, environmental factors (such as obesity, nutrition, and physical activity) modulate the phenotype. Most prominent is a variant of the transcription factor 7-like 2 gene that has been associated with type 2 diabetes in several populations. The mechanisms by which genetic alterations increase the susceptibility to type 2 diabetes are not clear, but they are predicted to alter insulin secretion. Investigation using genome-wide scanning for polymorphisms associated with type 2 DM is ongoing.

- Gestational Diabetes Mellitus (GDM) - Glucose intolerance may develop during pregnancy. Insulin resistance is related to the metabolic changes of late pregnancy and the increased insulin requirements may lead to IGT. Most women revert to normal glucose tolerance post-partum but have a substantial risk (30–60%) of developing DM later in life. Gestational diabetes mellitus (GDM) resembles type 2 diabetes in several respects, involving a combination of relatively inadequate insulin secretion and responsiveness. Even though it may be transient, untreated gestational diabetes can damage the health of the fetus or mother. Risks to the baby include macrosomia (high birth weight), congenital cardiac and central nervous system anomalies, and skeletal muscle

malformations. Increased fetal insulin may inhibit fetal surfactant production and cause respiratory distress syndrome. In fact, the rate of diabetes in expectant mothers has more than doubled in the past 6 years[5]

DIAGNOSIS

The National Diabetes Data Group and World Health Organization have issued diagnostic criteria for DM

- Symptoms of diabetes plus random blood glucose concentration ≥11.1 mmol/L (200 mg/dL) *or*
- Fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL) *or*
- Two-hour plasma glucose ≥ 11.1 mmol/L (200 mg/dL) during an oral glucose tolerance test

Glucose tolerance is classified into three categories based on the FPG

- FPG < 5.6 mmol/L (100 mg/dL) is considered normal;
- FPG = 5.6–6.9 mmol/L (100–125 mg/dL) is defined as IFG; and
- FPG ≥7.0 mmol/L (126 mg/dL) warrants the diagnosis of DM.

Based on the OGTT, IGT is defined as plasma glucose levels between 7.8 and 11.1 mmol/L (140 and 199 mg/dL) and diabetes is defined as a glucose > 11.1 mmol/L (200 mg/dL), 2 h after a 75-g oral glucose load. Some individuals have both IFG and IGT. Individuals with IFG and/or IGT, recently designated *pre-diabetes* by the American Diabetes Association (ADA), are at substantial risk for developing type 2 DM (25–40% risk over the next 5 years) and have an increased risk of cardiovascular disease.

The current criteria for the diagnosis of DM emphasize that the FPG is the most reliable and convenient test for identifying DM in asymptomatic individuals. A random plasma glucose concentration ≥11.1 mmol/L (200 mg/dL) accompanied by classic symptoms of DM (polyuria, polydipsia, weight loss) is sufficient for the diagnosis of DM .Some investigators have advocated the hemoglobin A1C as a diagnostic test for DM. Though there is a strong correlation between elevations in the plasma glucose and the HbA1C , the relationship between the FPG and the A1C in individuals with normal glucose tolerance or mild glucose intolerance is less clear and thus the use of the A1C is not currently recommended to diagnose diabetes.

TYPE 2 DIABETES MELLITUS

RISK FACTORS

- Family history of diabetes (i.e., parent or sibling with type 2 diabetes)
- Obesity (BMI ≥25 kg/m2)
- Habitual physical inactivity
- Previously identified IFG or IGT
- History of GDM or delivery of baby >4 kg (>9 lb)
- Hypertension (blood pressure≥140/90 mmHg)
- HDL cholesterol level <35 mg/dL (0.90 mmol/L) and/or a triglyceride level >250 mg/dL (2.82 mmol/L)
- Polycystic ovary syndrome or acanthosis nigricans
- History of vascular disease

PATHOPHYSIOLOGY

Type 2 DM is characterized by impaired insulin secretion, insulin resistance, excessive hepatic glucose production and abnormal fat metabolism. In the early stages of the disorder, glucose tolerance remains near-normal, despite insulin resistance, because the pancreatic beta cells compensate by increasing insulin output. As insulin resistance and compensatory hyperinsulinemia progress, the pancreatic islets in certain individuals are unable to sustain the hyperinsulinemic state. IGT, characterized by elevations in postprandial glucose, then develops. A further decline in insulin secretion and an increase in hepatic glucose production lead to overt diabetes with fasting hyperglycemia. Ultimately, beta cell failure may ensue.

METABOLIC ABNORMALITIES

Abnormal Muscle and Fat Metabolism

Insulin resistance, the decreased ability of insulin to act effectively on target tissues (especially muscle, liver, and fat), is a prominent feature of type 2 DM and results from a combination of genetic susceptibility and obesity. Insulin resistance impairs glucose utilization by insulin-sensitive tissues and increases hepatic glucose output; both effects contribute to the hyperglycemia. Increased hepatic glucose output predominantly accounts for increased FPG levels, whereas decreased peripheral glucose usage results in postprandial hyperglycemia. In skeletal muscle, there is a greater impairment in nonoxidative glucose usage (glycogen formation) than in oxidative glucose metabolism through glycolysis. Glucose metabolism in insulin-independent tissues is not altered in type 2 DM.

The precise molecular mechanism leading to insulin resistance in type 2 DM has not been elucidated. Insulin receptor levels and tyrosine kinase activity in skeletal muscle are reduced, but these alterations are most likely secondary to hyperinsulinemia and are not a primary defect. Therefore, "postreceptor" defects in insulin-regulated phosphorylation / dephosphorylation may play the predominant role in insulin resistance. For example, a PI-3-kinase signaling defect may reduce translocation of GLUT4 to the plasma membrane. Other abnormalities include the accumulation of lipid within skeletal myocytes, which may impair mitochondrial oxidative phosphorylation and reduce insulin-stimulated mitochondrial ATP production. Impaired fatty acid oxidation and lipid accumulation within skeletal myocytes may generate reactive oxygen species such as lipid peroxides. Of note, not all insulin signal transduction pathways are resistant to the effects of insulin (e.g., those controlling cell growth and differentiation using the mitogenic-activated protein kinase pathway). Consequently, hyperinsulinemia may increase the insulin action through these pathways, potentially accelerating diabetes-related conditions such as atherosclerosis.

The obesity accompanying type 2 DM, particularly in a central or visceral location, is thought to be part of the pathogenic process. The increased adipocyte mass leads to increased levels of circulating free fatty acids and other fat cell products. For example, adipocytes secrete a number of biologic products (nonesterified free fatty acids, leptin, resistin, and adiponectin). In addition to regulating body weight, appetite, and energy expenditure, adipokines also modulate insulin sensitivity. The increased production of free fatty acids and some adipokines

may cause insulin resistance in skeletal muscle and liver. For example, free fatty acids impair glucose utilization in skeletal muscle, promote glucose production by the liver and impair beta cell function. In contrast, the production by adipocytes of adiponectin, an insulin-sensitizing peptide, is reduced in obesity and this may contribute to hepatic insulin resistance. Adipocyte products and adipokines also produce an inflammatory state and may explain why markers of inflammation such as IL-6 and C-reactive protein are often elevated in type 2 DM.

Insulin secretion and sensitivity are interrelated. In type 2 DM, insulin secretion initially increases in response to insulin resistance to maintain normal glucose tolerance. Initially, the insulin secretory defect is mild and selectively involves glucose-stimulated insulin secretion. The response to other nonglucose secretagogues, such as arginine, is preserved. Eventually, the insulin secretory defect progresses to a state of grossly inadequate insulin secretion.

The reasons for the decline in insulin secretory capacity in type 2 DM is unclear. The assumption is that a second genetic defect—superimposed upon insulin resistance—leads to beta cell failure. Islet amyloid polypeptide or amylin is cosecreted by the beta cell and forms the amyloid fibrillar deposit found in the islets of individuals with long-standing type 2 DM. Whether such islet amyloid deposits are a primary or secondary event is not known. The metabolic environment of diabetes may also negatively impact islet function. For example, chronic hyperglycemia paradoxically impairs islet function ("glucose toxicity") and leads to a worsening of

hyperglycemia. Improvement in glycemic control is often associated with improved islet function. In addition, elevation of free fatty acid levels ("lipotoxicity") and dietary fat may also worsen islet function. Beta cell mass is decreased in individuals with long-standing type 2 diabetes.

Increased Hepatic Glucose and Lipid Production

In type 2 DM, insulin resistance in the liver reflects the failure of hyperinsulinemia to suppress gluconeogenesis, which results in fasting hyperglycemia and decreased glycogen storage by the liver in the postprandial state. Increased hepatic glucose production occurs early in the course of diabetes, though likely after the onset of insulin secretory abnormalities and insulin resistance in skeletal muscle. As a result of insulin resistance in adipose tissue and obesity, free fatty acid (FFA) flux from adipocytes is increased, leading to increased lipid [very low density lipoprotein (VLDL) and triglyceride] synthesis in hepatocytes. This lipid storage or steatosis in the liver may lead to nonalcoholic fatty liver disease and abnormal liver function tests. This is also responsible for the dyslipidemia found in type 2 DM [elevated triglycerides, reduced high-density lipoprotein (HDL), and increased small dense low-density lipoprotein (LDL) particles].

GESTATIONAL DIABETES

Gestational diabetes is defined as "any degree of glucose intolerance with onset or first recognition during pregnancy"[18]. This definition includes the possibility that patients may have previously undiagnosed diabetes mellitus, or may have developed diabetes coincidentally with pregnancy. It is usually diagnosed by screening during pregnancy. It is said to affect 3-10% of pregnancies, depending on

the population studied[16]. No specific cause has been identified, but it is believed that the hormones produced during pregnancy increase a woman's resistance to insulin, resulting in impaired glucose tolerance. Most patients are treated only with diet modification and moderate exercise, but some take anti-diabetic drugs, including insulin [17].

The White classification, named after Priscilla White[20] is widely used to assess maternal and fetal risk. It distinguishes between gestational diabetes (type A) and diabetes that existed prior to pregnancy (pregestational diabetes). These two groups are further subdivided according to their associated risks and management[21]

There are 2 subtypes of gestational diabetes (diabetes which began during pregnancy):

- *Type A1:* abnormal oral glucose tolerance test (OGTT) but normal blood glucose levels during fasting and 2 hours after meals; diet modification is sufficient to control glucose levels
- *Type A2:* abnormal OGTT compounded by abnormal glucose levels during fasting and/or after meals; additional therapy with insulin or other medications is required

RISK FACTORS

Classical risk factors for developing gestational diabetes are the following[22]:

- A previous diagnosis of gestational diabetes or prediabetes, impaired glucose tolerance, or impaired fasting glycaemia
- A family history revealing a first degree relative with type 2 diabetes
- Maternal age - a woman's risk factor increases as she gets older (especially for women over 35 years of age)

- Being overweight, obese or severely obese
- A previous pregnancy which resulted in a child with a high birth weight >4000 g

MECHANISMS BEHIND GDM

Insulin resistance is a normal phenomenon emerging in the second trimester of pregnancy, which progresses thereafter to levels seen in non-pregnant patients with type 2 diabetes. It is thought to secure glucose supply to the growing fetus. Women with GDM have an insulin resistance they cannot compensate with increased production in the β-cells of the pancreas. Placental hormones, and to a lesser extent increased fat deposits during pregnancy, seem to mediate insulin resistance during pregnancy. Cortisol, progesterone, human placental lactogen, prolactin and estradiol also play a role in insulin resistance[23]. The following mechanisms are some of the explanations for the development of GDM : autoimmunity, single gene mutations, obesity and other mechanisms[24].

Because glucose travels across the placenta (through diffusion facilitated by GLUT3 carriers), the fetus is exposed to higher glucose levels. This leads to increased fetal levels of insulin (insulin itself cannot cross the placenta). The growth-stimulating effects of insulin can lead to excessive growth and a large body (macrosomia).

SCREENING TESTS FOR GESTATIONAL DIABETES

- Non Challenge blood glucose tests
    - Fasting Glucose Test
    - 2-Hour Post Prandial glucose test
    - Random Glucose Test
- Screening Glucose Challenge Test
- Oral Glucose Tolerance Test (OGTT)

NON-CHALLENGE BLOOD GLUCOSE TESTS

When a plasma glucose level is found to be higher than 126 mg/dl (7.0 mmol/l) after fasting, or over 200 mg/dl (11.1 mmol/l) on any occasion, and if this is confirmed on a subsequent day, the diagnosis of GDM is made and no further testing is required[19]

SCREENING GLUCOSE CHALLENGE TEST

The screening glucose challenge test (sometimes called the O'Sullivan test) is performed between 24–28 weeks, and can be seen as a simplified version of the oral glucose tolerance test (OGTT). It involves drinking a solution containing 50 grams of glucose, and measuring blood levels 1 hour later[19].

If the cut-off point is set at 140 mg/dl (7.8 mmol/l), 80% of women with GDM will be detected. If this threshold is lowered to 130 mg/dl, 90% of GDM cases will be detected, but there will also be more women who will be subjected to a consequent OGTT unnecessarily.

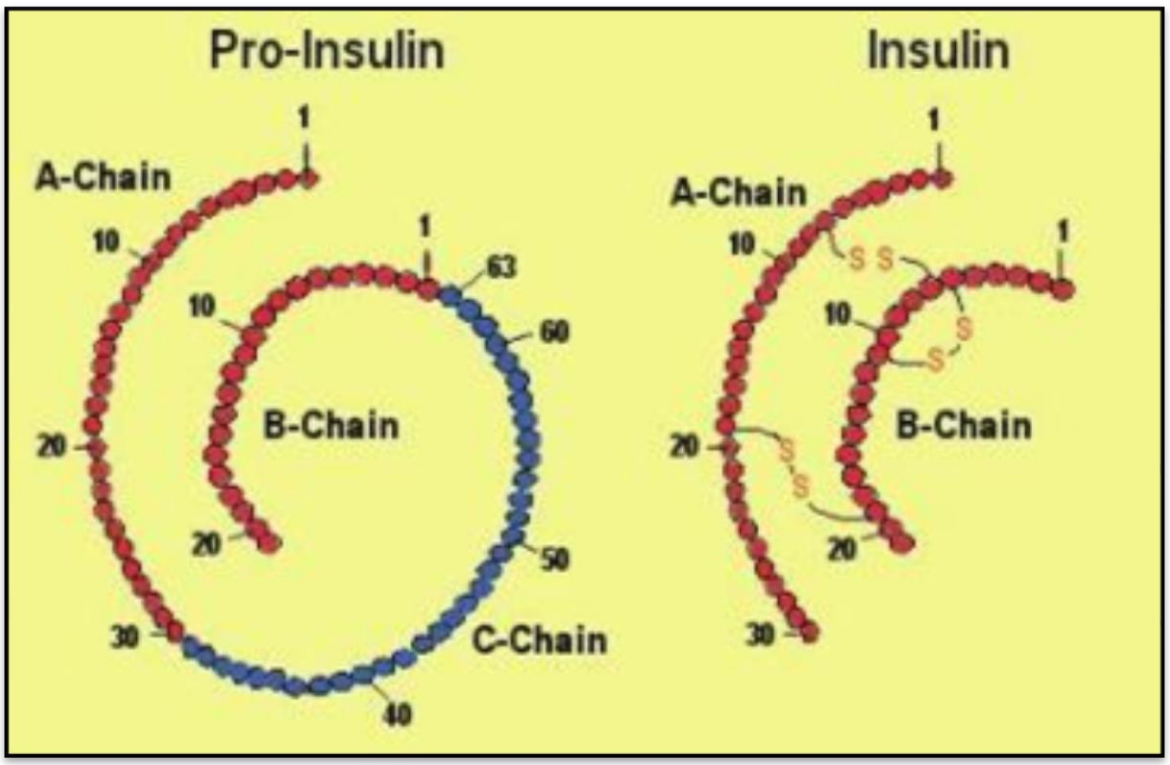


Figure 1 – Structure of Insulin and Proinsulin

ORAL GLUCOSE TOLERANCE TEST

The following are the values which the American Diabetes Association considers to be abnormal during the 100 g of glucose OGTT:

- Fasting blood glucose level ≥95 mg/dl (5.33 mmol/L)
- 1 hour blood glucose level ≥180 mg/dl (10 mmol/L)
- 2 hour blood glucose level ≥155 mg/dl (8.6 mmol/L)
- 3 hour blood glucose level ≥140 mg/dl (7.8 mmol/L)

An alternative test uses a 75 g glucose load and measures the fasting blood glucose levels and the levels after 1 and 2 hours, using the same reference values. This test will identify less women who are at risk, and there is only a weak concordance between this test and a 3 hour 100 g test.

INSULIN

Insulin is a peptide hormone composed of 51 amino acids and has a molecular weight of 5808 Da. It is produced in the islets of Langerhans in the pancreas. The name comes from the Latin word insula for "island". The proinsulin precursor of insulin is encoded by the INS gene.

STRUCTURE

Insulin (Figure 1) is produced and stored in the body as a hexamer, while the active form is the monomer. The hexamer is an inactive form with long-term stability, which serves as a way to keep the highly reactive insulin protected, yet readily available. The C-Peptide of proinsulin however, differs much more amongst species, it is also a hormone, but a secondary one.

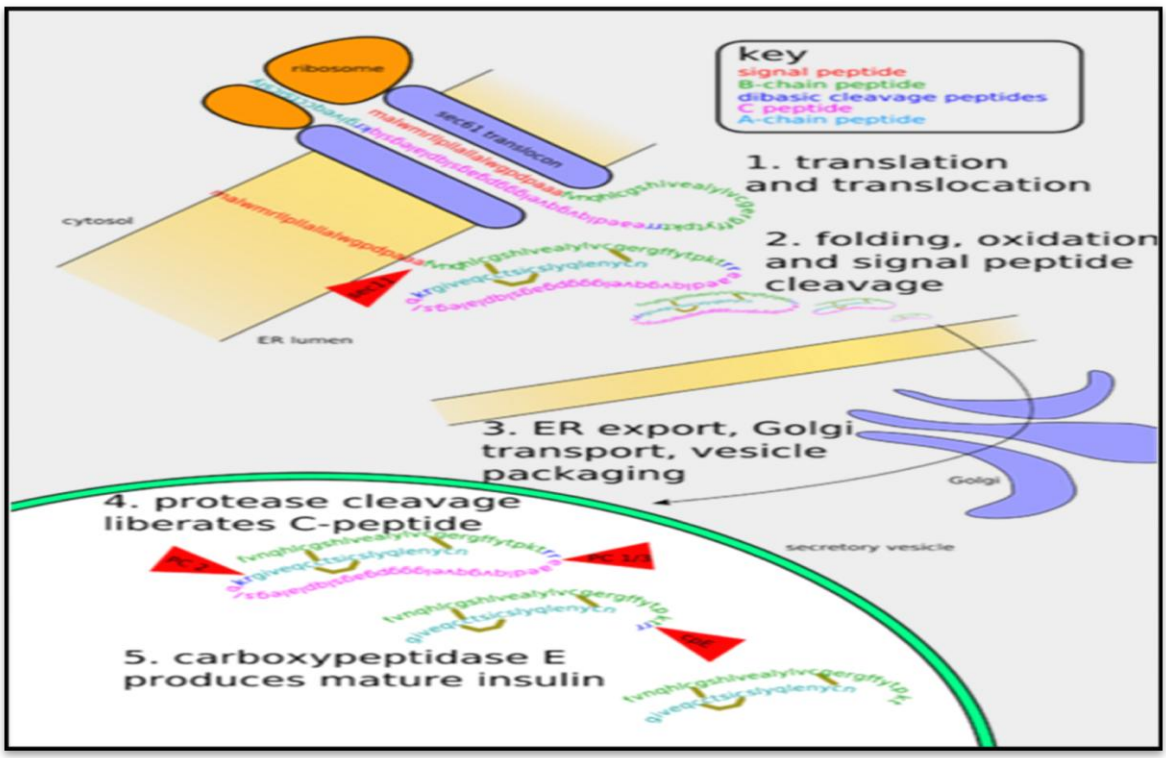


Figure 2 - Insulin Biosynthesis

BIOSYNTHESIS

Insulin is produced in the beta cells of the pancreatic islets ((Figure 2). It is initially synthesized as a single-chain 86-amino-acid precursor polypeptide, preproinsulin. Subsequent proteolytic processing removes the aminoterminal signal peptide, giving rise to proinsulin. Proinsulin is structurally related to insulin-like growth factors I and II, which bind weakly to the insulin receptor. Cleavage of an internal 31-residue fragment from proinsulin generates the C-peptide and the A (21 amino acids) and B (30 amino acids) chains of insulin, which are connected by disulfide bonds. The mature insulin molecule and C-peptide are stored together and cosecreted from secretory granules in the beta cells. Because the C-peptide is cleared more slowly than insulin, it is a useful marker of insulin secretion and allows discrimination of endogenous and exogenous sources of insulin in the evaluation of hypoglycemia. Pancreatic beta cells cosecrete islet amyloid polypeptide (IAPP) or amylin, a 37-amino-acid peptide, along with insulin. The role of IAPP in normal physiology is unclear, but it is the major component of the amyloid fibrils found in the islets of patients with type 2 diabetes and an analogue is sometimes used in treating both type 1 and type 2 DM.

The endogenous production of insulin is regulated in several steps along the synthesis pathway:

- At transcription from the insulin gene
- In mRNA stability
- At the mRNA translation and

In the post translational modifications

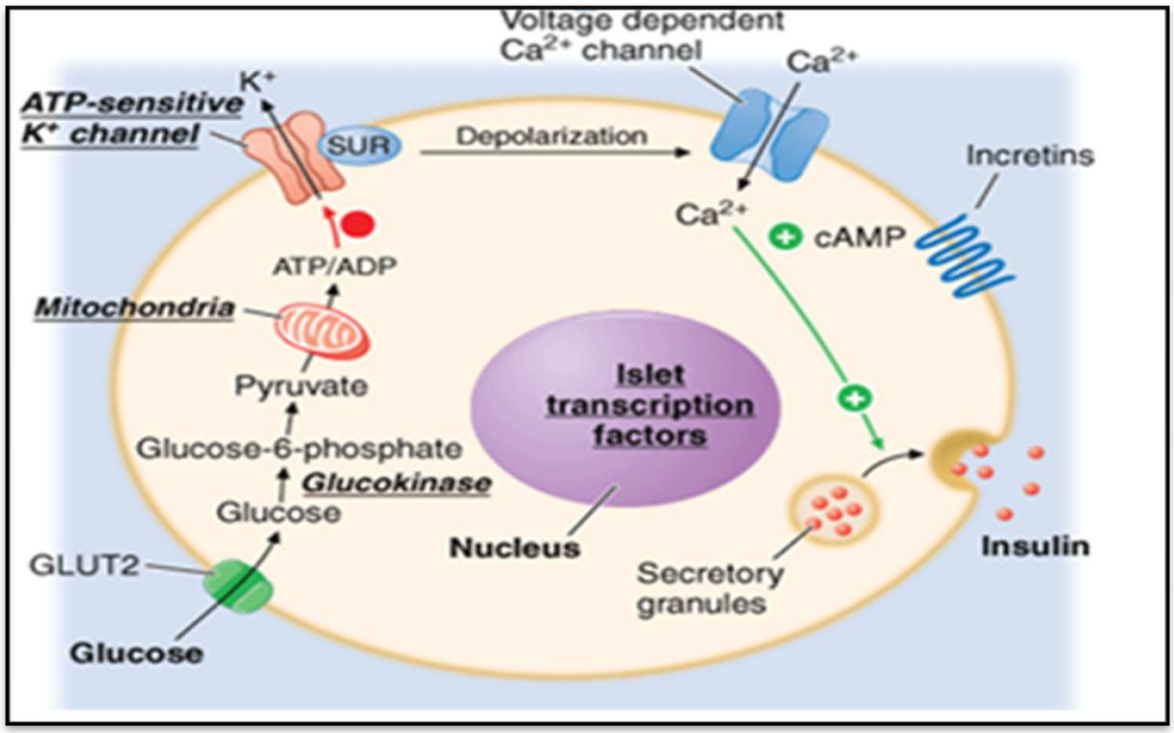


Figure 3 - Mechanism of Insulin Release

RELEASE

Beta cells in the islets of Langerhans release insulin in two phases. The first phase insulin release is rapidly triggered in response to increased blood glucose levels. The second phase is a sustained, slow release of newly formed vesicles that are triggered independently of sugar. The description of first phase release is as follows ((Figure 3):

- Glucose enters the beta cells through the glucose transporter GLUT2
- Glucose goes into glycolysis and the respiratory cycle where multiple high-energy ATP molecules are produced by oxidation
- Dependent on the ATP:ADP ratio and hence blood glucose levels, the ATP-dependent potassium channels (K+) close and the cell membrane depolarizes
- On depolarization, voltage controlled calcium channels (Ca^{2+}) open and calcium flows into the cells
- An increased calcium level causes activation of phospholipase C, which cleaves the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-triphosphate and diacylglycerol.
- Inositol 1,4,5-triphosphate (IP3) binds to receptor proteins in the membrane of endoplasmic reticulum (ER). This allows the release of Ca^{2+} from the ER via IP3 gated channels, and further raises the cell concentration of calcium.
- Significantly increased amounts of calcium in the cells causes release of previously synthesized insulin, which has been stored in secretory vesicles

ACTION

Insulin binding to its receptor stimulates intrinsic tyrosine kinase activity, leading to receptor autophosphorylation and the recruitment of intracellular signaling

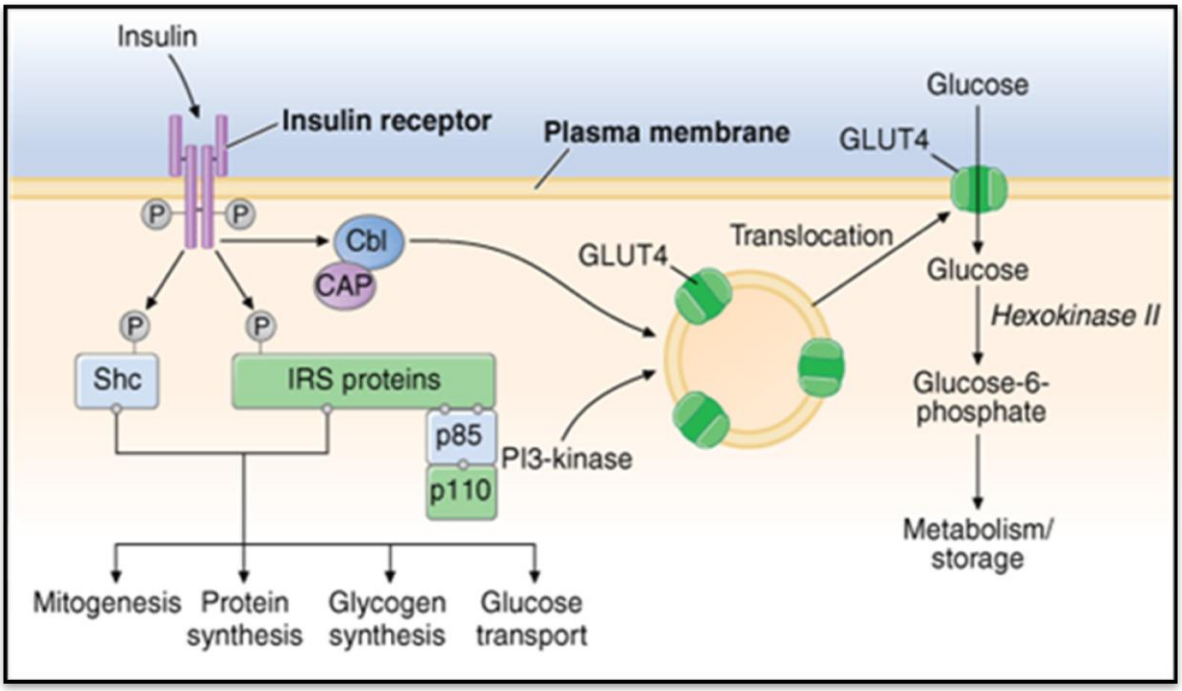


Figure 4 - Insulin Signal Transduction Pathway in Skeletal Muscle

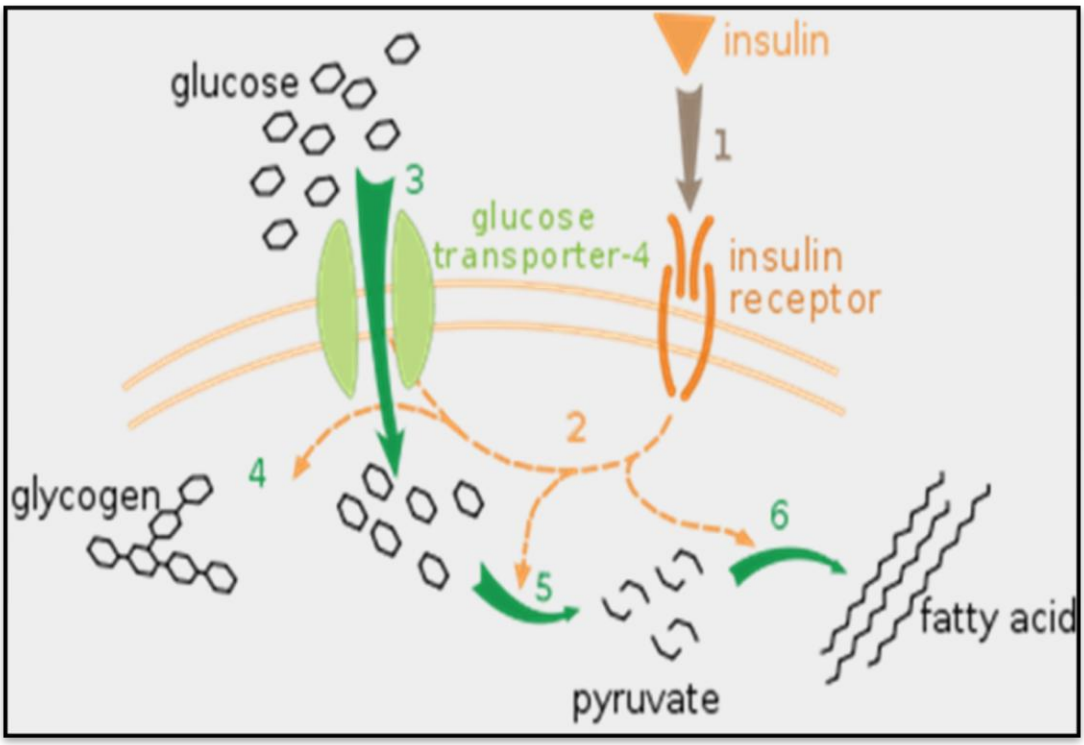


Figure 5 - Effect of Insulin on Glucose Metabolism

molecules, such as insulin receptor substrates (IRS). IRS and other adaptor proteins initiate a complex cascade of phosphorylation and dephosphorylation reactions, resulting in the widespread metabolic and mitogenic effects of insulin (Figure 4). As an example, activation of the phosphatidylinositol-3'-kinase (PI-3-kinase) pathway stimulates translocation of glucose

transporters (e.g., GLUT4) to the cell surface, an event that is crucial for glucose uptake by skeletal muscle and fat. Activation of other insulin receptor signaling pathways induces

glycogen synthesis, protein synthesis, lipogenesis and regulation of various genes in insulin-responsive cells.

EFFECT OF INSULIN ON GLUCOSE METABOLISM

Insulin binds to its receptor, which in turn starts many protein activation cascades. These include translocation of Glut-4 transporter to the plasma membrane and influx of glucose, glycogen synthesis, glycolysis and fatty acid synthesis (Figure 5).

DEGRADATION

It has been estimated that an insulin molecule produced endogenously by the pancreatic beta cells is degraded within approximately one hour after its initial release into circulation (insulin half-life ~ 4–6 minutes)[11,12].

C-PEPTIDE

C-peptide was first described in 1967 in connection with the discovery of the insulin biosynthesis[13]. It serves as an important linker between the A and the B chains of insulin and facilitates the efficient assembly, folding, and processing of insulin in

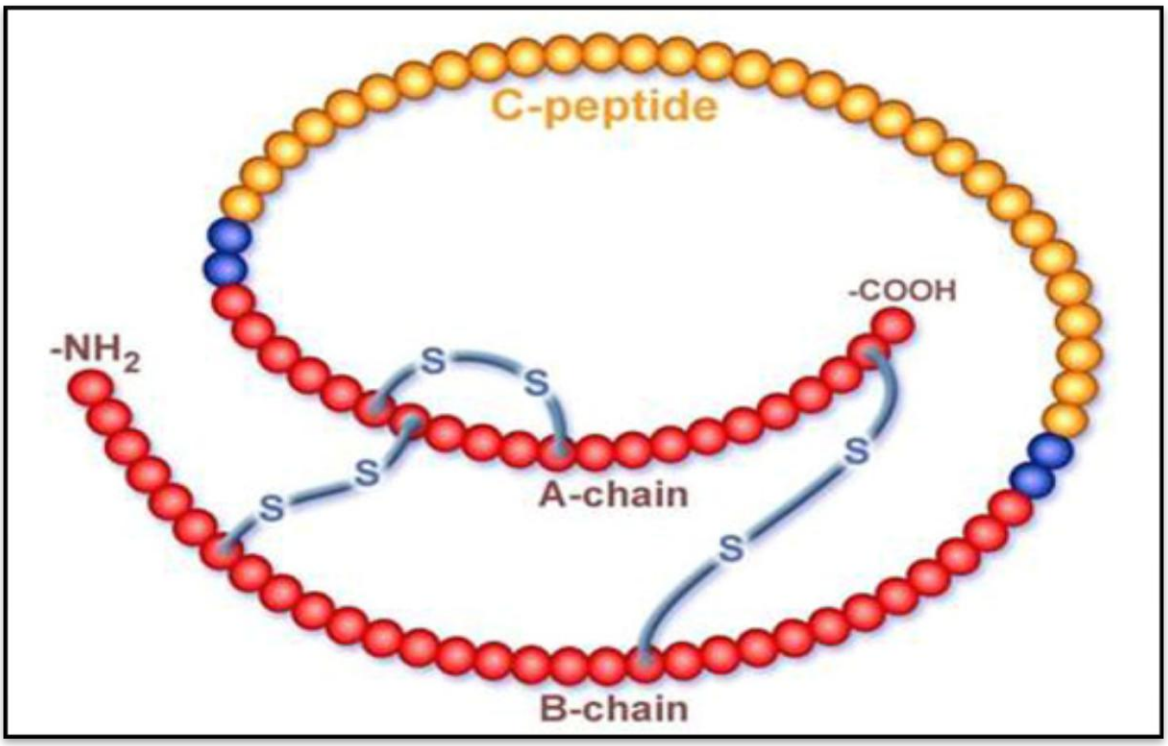


Figure 6 - Structure of C-peptide

the endoplasmic reticulum. Equimolar amounts of C-peptide and insulin are then stored in secretory granules of the pancreatic beta cells and both are eventually released to the portal circulation. Initially, the sole interest in C-peptide was as a marker of insulin secretion and it has been of great value in further understanding of the pathophysiology of type 1 and type 2 diabetes. The first documented use of the C-peptide test was in 1972. During the past decade, however, C-
peptide has been found to be a bioactive peptide in its own right, with effects on microvascular blood flow and tissue health (Figure 6).

FUNCTION

C-peptide has been shown to bind to the surface of a number of cell types such as neuronal, endothelial, fibroblast and renal tubular, at nanomolar concentrations to a receptor that is likely G-protein coupled. The signal activates Ca2+ dependent intracellular signaling pathways such as MAPK, PLCγ and PKC, leading to upregulation of a range of transcription factors as well as eNOS and Na+K+ATPase activities[14]. The latter two enzymes are known to have reduced activities in patients with type I diabetes and have been implicated in the development of long-terms complications of type I diabetes such as peripheral and autonomic neuropathy. In vivo studies in animal models of type 1 diabetes have established that C-peptide administration results in significant improvements in nerve and kidney function. C-peptide has also been reported to have anti-inflammatory effects and it aids in repair of smooth muscle cells[15].

LEPTIN

Leptin (Greek *leptos* meaning thin) is a 16 kDa protein hormone that plays a key role in regulating energy intake and energy expenditure, including appetite and

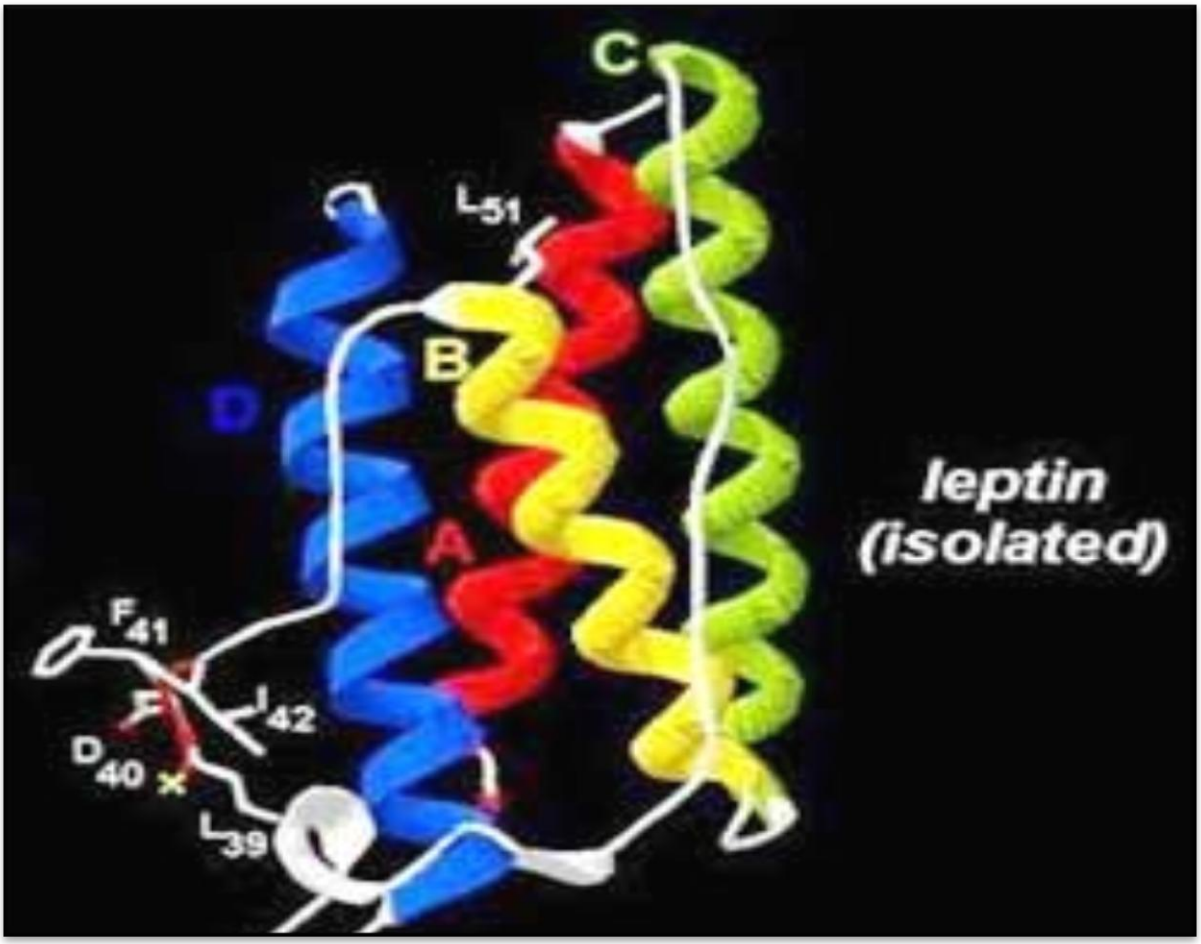


Figure 7 - Structure of Leptin

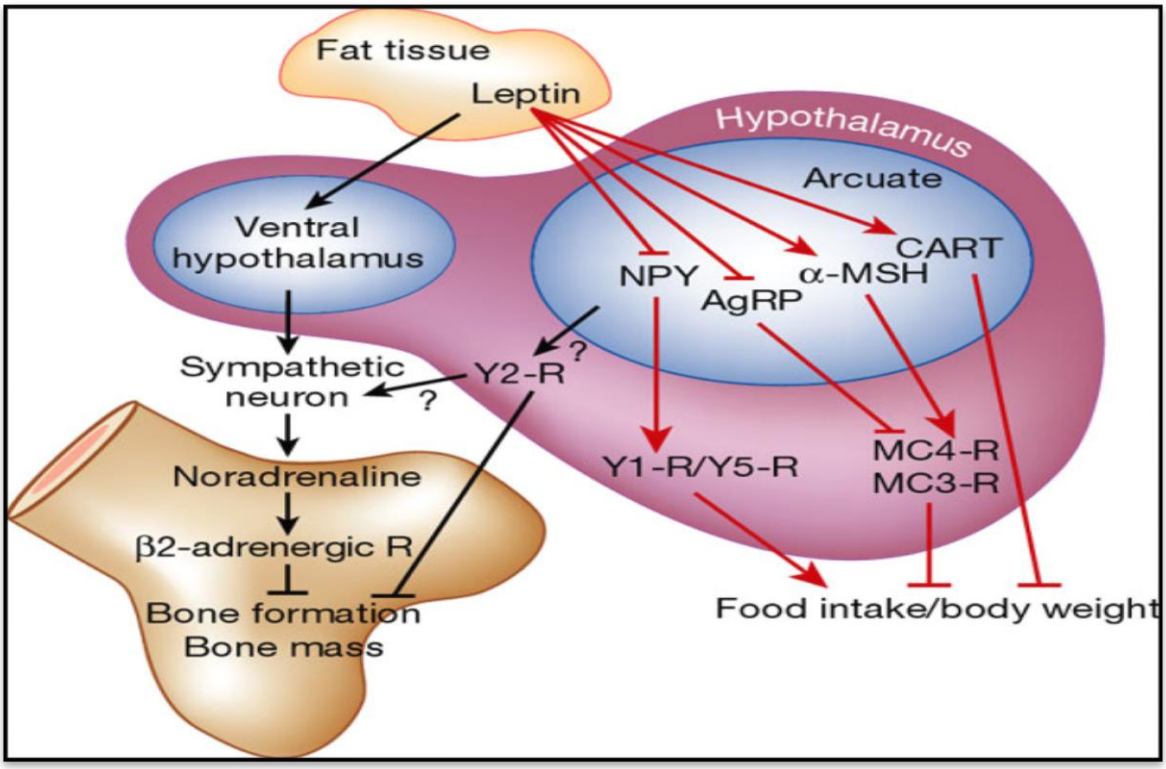


Figure 8 - Central Connections of Leptin

metabolism. It is one of the most important adipose derived hormones[25] (Figure 7). The Ob(Lep) gene (Ob for obese, Lep for leptin) is located on chromosome 7 in humans[26].

DISCOVERY

The effects of leptin were observed by studying mutant obese mice that arose at random within a mouse colony at the Jackson Laboratory in 1950[27]. These mice were massively obese and excessively voracious. Ultimately, several strains of laboratory mice have been found to be homozygous for single-gene mutations that causes them to become grossly obese, and they fall into two classes: "ob/ob", those having mutations in the gene for

the protein hormone leptin and "db/db", those having mutations in the gene that encodes the receptor for leptin. When ob/ob mice are treated with injections of leptin, they lose their excess fat and return to normal body weight. Leptin was discovered in 1994 by Jeffrey M. Friedman and colleagues at the Rockefeller University through the study of such mice[28].

ACTIONS ON HYPOTHALAMUS

Leptin acts on receptors in the hypothalamus of the brain where it inhibits appetite by

- Counteracting the effects of neuropeptide Y (a potent feeding stimulant secreted by cells in the gut and in the hypothalamus)
- Counteracting the effects of anandamide (another potent feeding stimulant)
- Promoting the synthesis of α-MSH, an appetite suppressant (Figure 8)

The absence of leptin (or its receptor) leads to uncontrolled food intake and resulting obesity. Thus, circulating leptin levels give the brain, input regarding

energy storage . Leptin works by inhibiting the activity of neurons that contain neuropeptide Y (NPY) and agouti-related peptide (AgRP) and by increasing the activity of neurons expressing α-melanocyte-stimulating hormone (α-MSH). The NPY neurons are a key element in the regulation of appetite; small doses of NPY injected into the brains of experimental animals stimulates feeding, while selective destruction of the NPY neurons in mice causes them to become anorexic. Conversely, α-MSH is an important mediator of satiety and differences in the gene for the receptor at which α-MSH acts in the brain are linked to obesity in humans.

SEXUAL DIMORPHISM OF LEPTIN

Sexual dimorphism of leptin in humans is evident in both *ob* mRNA expression[29,30] and in the correlation between leptin concentrations and fat mass [31,32]. Kennedy and co-workers[32] proposed that the observed gender differences in leptin synthesis are because of the stimulating roles of oestrogens and/or a suppressive effect of circulating androgens, while other authors have been unable to find a correlation between sexual dimorphism of leptin and sex hormones.

SYNTHESIS

Leptin is translated as a 167 amino acid protein with an amino-terminal secretory signal sequence of 21 amino acids. The signal sequence is functional and results in the trans-location of leptin into microsomes with the subsequent removal of the signal peptide. Therefore, leptin circulates in the blood as protein of 146 amino acid residues.

It is manufactured primarily in the adipocytes of white adipose tissue and the level of circulating leptin is directly proportional to the total amount of fat in the

body. In addition to white adipose tissue—the major source of leptin—it can also be produced by brown adipose tissue, placenta (syncytiotrophoblasts), ovaries, skeletal muscle, stomach (lower part of fundic glands), mammary epithelial cells, bone marrow, pituitary and liver.[33] Leptin has also been discovered to be synthesised from gastric chief cells in the stomach[34].

SITES OF LEPTIN SYNTHESIS

WHITE ADIPOSE TISSUE

White adipose tissue (WAT) is the main site of leptin synthesis, but it is now evident that the hormone is produced in other tissues as well. There is some evidence that brown adipose tissue (BAT) is also a site of leptin production[35-42]. A major issue is the physiological role of leptin produced by brown fat. If the ob gene is expressed by brown adipocytes, then there are important implications for the physiological role of leptin. One possibility is that it simply adds to the pool of circulating hormone and thus its contribution would be small relative to that of white fat in mature animals and man[43]. Other reports suggest that the expression that is observed may be a reflection of contamination or infiltration of brown fat with white adipocytes[44]. However, Moinat *et al*[35] reported that the level of mRNA in interscapular brown fat was about 40% of that in the epididymal white fat depot, a result that is unlikely to be explained by infiltration or contamination. If the *ob* gene is expressed by brown adipocytes, then there are important implications for the physiological role of leptin.

Comparison of the levels of *ob* mRNA in different adipose tissue depots suggests that there are site-specific variations in the expression of the *ob* gene in both rodents[44,45] and humans[47,48]. In humans[46], leptin expression appears to be greater in subcutaneous than in omental adipose tissue[47,48]. The differences in mRNA level

between various adipose tissue sites may reflect differences in fat cell size; the larger the adipocytes, the greater the expression of the *ob* gene[44,45,46,49]. However, Lonnqvist *et al* using *in situ* hybridization, found no differences in leptin expression between subcutaneous and omental fat in a small group of four obese humans.

In rodents the opposite is apparent as leptin expression may be lower in subcutaneous fat than in the internal depots and the highest level of expression is generally evident in the epididymal (males) and perirenal adipose tissues[45,47]. However, site-specific leptin expression also varies with maturation as in adult rodents; the *ob* mRNA level is much higher in the gonadal and perirenal sites than in subcutaneous depots,[44] but in suckling rats during the first 2 weeks after birth, the subcutaneous fat is the main site of *ob* gene expression.

PLACENTA

An important new dimension to leptin biology has emerged with the recognition that the placenta and ovary express the leptin gene and that they are sites of production of the hormone. This has been demonstrated in mice, rats and humans[50-53].

Two hypotheses about the role of placental synthesis of leptin are that it may either be a new growth factor, or act as a signal of energy status between the mother and the fetus. The placenta also expresses the leptin receptor gene, implying that the organ is a target for the action of leptin in addition to being a source of the hormone[50]. These observations suggest that leptin may act in an autocrine manner. The

expression of leptin by syncytiotrophoblasts[50,55,56] has added support to the hypothesis of the importance of leptin in nutrient transfer.

FETAL TISSUES

In situ hybridization and immunohistochemical studies on pregnant mice have demonstrated leptin synthesis in several regions of the fetus, including the heart, bone and cartilage, choroid plexus of the fetal brain, lung, kidney, heart and liver and the cells of the hair follicle[57].

LIVER

Studies have reported that leptin gene expression in chicken was not only localized in adipose tissue, as in mammals, but also in liver. The expression of leptin in liver may be associated with a key role of this organ in avian species in controlling lipogenesis. The diverse picture of where leptin is produced in the body indicates that the functions of the hormone may extend beyond the basic lipostatic model

REGULATION OF LEPTIN SYNTHESIS

ENERGY EXCESS

The level of ob mRNA in white adipose tissue and the circulating leptin concentration are increased markedly in obesity, as shown in both human studies and in studies of several types of obese animals[46,58,59,60]. Indeed, in human subjects there is a high correlation between body mass index (BMI) and circulating leptin[46]. Thus, the greater the amount of adipose tissue, the higher the level of the hormone. In addition, adipocyte size appears to be another major determinant of leptin mRNA expression[61]. Hormonal factors have also been studied in relation to leptin and are discussed below.

FASTING

Changes in physiological state induced by fasting result in reduced ob gene expression and a subsequent fall in circulating leptin[44,62,63]. Rapid decreases in leptin levels in response to energy restriction or fasting appear to be greater than the decrease in fat mass in both rodents[58] and humans[64].

Thus, leptin may serve as a sensor of short-term changes in energy stores. Another study in mice has shown that re-feeding after fasting led to a rapid restoration of gene expression and plasma leptin[66]. The inhibitory effect of fasting on leptin expression might also be mediated by the sympathetic nervous system and the β3-AR.

REGULATION OF LEPTIN GENE EXPRESSION

The sympathetic nervous system, especially the β-AR axis, has been implicated in the regulation of leptin gene expression. For example, the β-AR agonists, noradrenaline and isoprenaline decreased leptin gene expression in WAT in mice[65] and decreased serum leptin.[67] It has been shown, both *in vivo* and *in vitro*, that β_3-agonists induce a decrease in the expression of leptin in WAT and BAT and decrease serum leptin.[43,65,68,69,70] The significance of β_3-AR in the control of leptin production in human subjects is uncertain, although there is evidence that the receptor may play an important role in the control of lipolysis in human omental and subcutaneous adipose tissue[71,72]. Mantzoros *et al*[68] reported that acute treatment of mice with the β_3-agonist, suppressed leptin expression in WAT. Thus it appears that the leptin response to specific β_3-AR activation is analogous to classical adrenergic activation. In addition, leptin has been shown to increase noradrenaline turnover in

thermogenic interscapular BAT[73] and to also increase the sympathetic outflow to other tissues[74,75].

ROLE OF LEPTIN IN ENERGY EXPENDITURE

Leptin is involved in increased energy utilization, possibly through enhanced thermogenesis mediated by tissue uncoupling proteins (UCPs). Studies on UCP subtype expression in rodents have shown that UCP-1 is expressed at higher levels during cold exposure and decreased by fasting[76] and that leptin treatment enhances[77] or does not alter BAT UCP-1 mRNA, compared with untreated controls.

OB-RECEPTOR (LONG AND SHORT FORMS)

Leptin interacts with six types of receptors (Ob-Ra–Ob-Rf, or LepRa-LepRf) which in turn are encoded by a single gene, LEPR[80]. Ob-R exhibit a widespread distribution including liver, heart, kidneys, lungs, small intestine, testes, ovaries, spleen, pancreas and adipose tissue[79,50,81,82,83,84.85]. However, analysis has shown that the majority of transcripts detected are those encoding short intracellular domain forms[81,82]. Within the hypothalamus, the long form leptin receptor has been found in several hypothalamic nuclei, including the arcuate nucleus, ventromedial, dorsomedial, lateral hypothalamic nuclei and the paraventricular nucleus.

All isoforms of the murine leptin receptor share an identical extracellular, ligand-binding domain of 816 amino acids, while the intracellular domain at the C-terminus is different[86,79] Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Rf contain transmembrane domains of 34 amino acids, whereas Ob-Re is truncated before the membrane-spanning domain and is therefore likely to be secreted.[79] Secreted

extracellular domains of other cytokine receptors are also known to function as specific binding proteins.[88] Ob-Rb (the human form of which is also referred to as Ob-RL; L=long) has a long cytoplasmic domain of 320 amino acids. Recently, another splice variant of leptin receptor, B219, was cloned from murine fetal liver.[89] Ob-Rb is the only receptor isoform that can signal intracellularly via the Jak-Stat and MAPK signal transduction pathways[87]

Ob-Receptor induced signal transduction

Upon leptin binding to its receptor, receptor dimerisation occurs, which seems to be necessary for signaling activity[90,91,92]. After leptin binding and receptor homodimerization, the long form of the receptor (Ob-Rb) activates JAK/STAT pathways. The activated JAKs phosphorylate tyrosine sites on the intracellular domain of the receptor, which serve as docking sites for the Src-homology domains (SH2, SH3) that occur in all of the STATs[86,93]. The phosphorylated intracellular domain then provides a binding site for a STAT protein. The activated STAT proteins dimerise and translocate to the nucleus, where they bind DNA and activate transcription. Ob-Rb activation may also phosphorylate JAK leading to the activation of insulin receptor substrate (IRS-1) and mitogen-activated protein kinase (MAPK). Upon leptin binding, the short form of the leptin receptor (OB-Ra) phosphorylates IRS-1 and consequently activates MAPK. The activation of MAPK leads to the activation of pp90 S6-K[95,96].

Recently, a new family of cytokine-inducible inhibitors of signaling has been identified, including cytokine-inducible sequence (CIS) and suppressor-of-cytokine-signalling-3 (SOCS3)[97,98-103] CIS and SOCS3 may function as part of an intracellular

negative-feedback loop, inhibiting JAK activity, and thereby switching off or dampening cytokine signal transduction.[101,102]

PERIPHERAL INTERACTIONS OF LEPTIN

ADIPOSITY SIGNAL

- Leptin and insulin are known to act as an adiposity signal
- Leptin circulates at levels proportional to body fat
- It enters the central nervous system (CNS) in proportion to its plasma concentration
- Its receptors are found in brain neurons involved in regulating energy intake and expenditure
- It controls food intake and energy expenditure by acting on receptors in the mediobasal hypothalamus[104]

INTERACTION WITH AMYLIN

Co-administration of two neurohormones known to have a role in body weight control, amylin (produced by beta cells in the pancreas) and leptin (produced by fat cells), results in sustained, fat-specific weight loss in a leptin-resistant animal model of obesity.[105]

LEPTIN AND HAEMOPOIESIS

It has recently been shown that Ob-R is expressed in haemopoietic cells and that leptin may be linked to the proliferation and differentiation of haemopoietic precursors.[89,106] It is perhaps not surprising that leptin appears to have a role in haemopoiesis. Leptin structure is similar to the cytokines, containing four alpha helices. Furthermore, the Ob-R is a member of the type I cytokine receptor

superfamily. In cell culture studies, it appears that leptin has a mediation role, at least in murine myelocytic cells from bone marrow, in cell proliferation.[107]

Studies have reported that relatively high concentrations of human leptin corresponding to plasma leptin levels in obese individuals, promoted platelet aggregation. At lower concentrations, leptin failed to potentiate agonist-induced platelet aggregation. This suggests that the leptin effect of potentiating platelet aggregation operates specifically in obese individuals and it may be a key coupling factor between obesity and the cardiovascular disease associated with syndrome X (which includes glucose intolerance, insulin insensitivity and abnormal fat distribution) and diabetes. Alternatively, these data may indicate that leptin action in platelets functions via an Ob-R-independent mechanism.

LEPTIN AND ANGIOGENESIS

Further roles for leptin have been proposed in the circulatory system, including stimulation of endothelial cell growth and angiogenesis.[108,109] Several studies have demonstrated that vascular endothelium expresses the long form of the receptor[110,111,108,109,112] (in rodents and humans[112]) and short[101,113,114] form of the leptin receptor in rodents.

LEPTIN AND REPRODUCTION

Human ovary and prostate and murine ovary and embryo express mRNA for the leptin receptor.[86,89,115] Although much of the evidence suggests a central site for the effects of leptin on the reproductive axis, a direct effect on the ovary has also been demonstrated, as leptin at physiological concentrations inhibits insulin-induced oestradiol production by granulosa cells from both small and large bovine

follicles.[51,54,116] This is an example of non-competitive antagonism between leptin and insulin, demonstrated in a range of tissues. Expression of Ob-R has also been demonstrated in human granulosa cells.[117] Furthermore, Lindheim *et al*[118] showed that a significant increase in circulating leptin levels occurred during controlled ovarian hyperstimulation, suggesting that leptin plays a role in follicular growth and maturation.

To date, human and animal studies suggest that placental leptin is likely to affect maternal, placental and fetal function through both autocrine and paracrine mechanisms. It is possible that placental leptin may have physiological effects on the placenta including angiogenesis, growth and immunomodulation.[119] Leptin may also be involved in regulation of fetal and uterine metabolism.

LEPTIN IN FERTILITY

Hoggard and co-workers[57] found that reproductive hormone levels are reduced in *ob*/*ob* females, suggesting a functional defect in the hypothalamic pituitary axis. Several independent reports have demonstrated that correction of leptin deficiency in *ob*/*ob* mice by peripheral injections of recombinant leptin activates the reproductive axis and restores fertility in both sexes.

Furthermore, *in vitro* studies have revealed that oestrogens increase leptin mRNA expression.[30,121] Testosterone, in contrast, inhibits leptin gene expression *in vivo* and *in vitro*,[30,122] possibly through a direct suppressive effect. Studies have also suggested that androgens and oestrogens modulate leptin expression at the mRNA level through sex steroid receptor-dependent transcriptional mechanisms.[30] The study

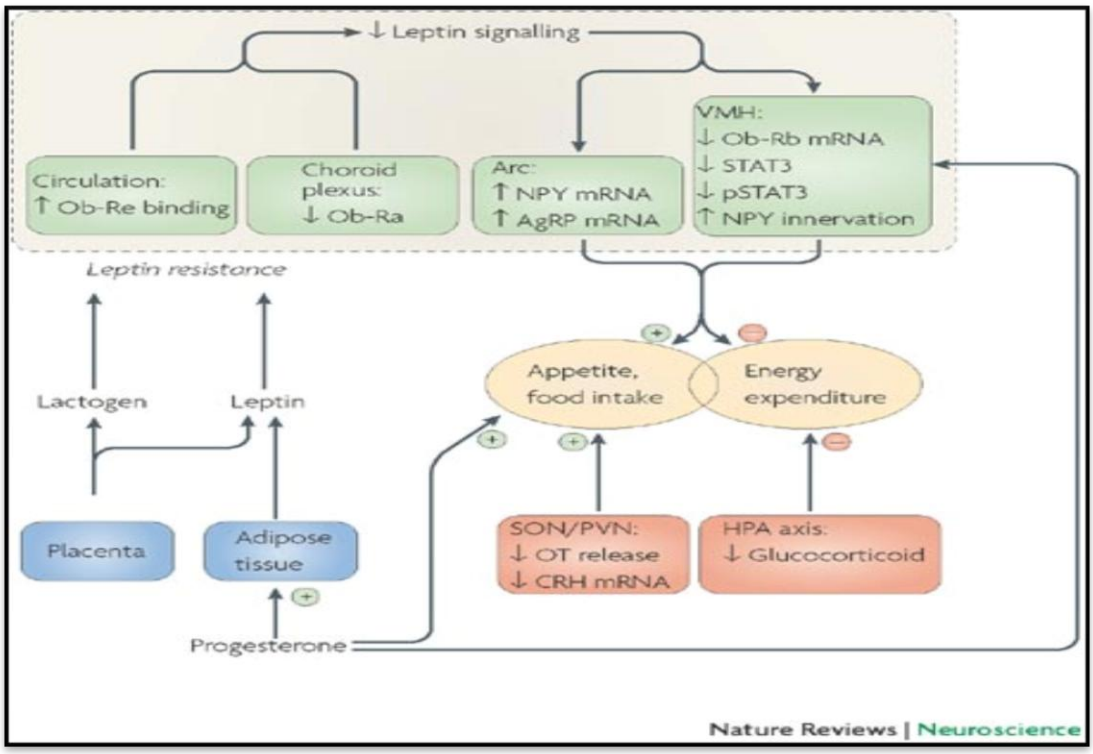


Figure 9 – Hormonal Regulation of Energy Balance in Pregnancy

of Jockenhovel *et al*[123] indicated that testosterone substitution normalized elevated serum leptin levels in hypogonadal men. These investigators have concluded that interaction of testosterone and leptin might be part of a hypothalamic /pituitary / gonadal/adipose tissue axis that is involved in body weight maintenance and reproductive function.

LEPTIN IN PREGNANCY

In females of most mammalian species, high leptin levels may signal the attainment of the sufficient long-term energy stores that are crucial for successful reproduction (Figure 9).[124] During human pregnancy, leptin levels were elevated throughout compared with the non-pregnant state.[53,125,126] Furthermore, in both humans[53] and mice,[124] serum leptin was particularly elevated during the second and third trimesters.

Both the short and the long forms of leptin receptor were detected in the human placenta in early and in the full-term stages of gestation, using quantitative RT-PCR.[127] Using *in situ* hybridization and immunohistochemistry, they also found that Ob-Rb was expressed by the apical membrane of the syncytiotrophoblasts.

Hyperleptinemia of Pregnancy

At present there appears to be a number of possible explanations for the increase in leptin levels in pregnancy: increased production by maternal fat; increased expression by the placenta; and increased levels of binding protein(s) (also of placental origin in the case of the mouse[128]), in the maternal circulation.[57] However, hyperleptinaemia during pregnancy is not associated with decreased food intake or a

decline in metabolic efficiency, as might be expected given one of the roles of leptin is as a satiety factor. Explanations for this may be a possible pregnancy-induced state of leptin resistance, or a change in leptin bioavailability.[129,130] It is interesting to note that some authors believe that the increase in leptin concentration during pregnancy is counter-intuitive as this is a period of increased nutritional demands and not one in which the actions of a satiety factor are expected to increase.[131] Others have hypothesized that the soluble form of the leptin receptor mediates leptin actions during pregnancy, as the circulating concentrations of this protein are increased, especially in the mouse,[128] although much less so in humans.[133] Given the range of interactions between leptin and other hormone axes, and the complex pattern of central and peripheral pathways invoked, we hypothesize that, if leptin does in fact have a role as a satiety factor, this role is centrally mediated and may not be significantly modulated by the soluble leptin receptor. Interactions such as those between leptin and insulin, which modulate oxidation of FFA and lipogenesis as part of the process which mobilizes energy reserves during pregnancy, are peripherally modulated and more influenced by the balance of energy reserve status and the demands of the fetus. Studies on the interaction of leptin binding protein status with plane of nutrition and energy reserve status during pregnancy will be required to confirm or refute this.

Studies suggest the possibility that leptin may be important for optimizing pregnancy outcome. Potential roles of leptin may include facilitation of endocrine responses to pregnancy, maintenance of maternal fuel homeostasis during a period of increased nutrient intake and requirement and/or optimization of fetal growth and development.The relative contributions of fetal-derived leptin and maternal leptin to

maternal total leptin has recently been described by Linnemann *et al*[134] using an *in vitro* perfusion technique. This study suggests that only 1-2% of placental leptin enters the fetal circulation, while approximately 98% enters the maternal circulation. Although not yet determined, the interaction of leptin with insulin may also be important in pregnancy. Leptin appears to act as a permissive factor with respect to its effects on insulin-mediated fuel storage in muscle and in the liver and on insulin-mediated fuel utilization by muscle (see below). Thus, one of the actions of fetal- and placental-derived leptin may be to act on maternal liver and muscle, changing the dynamics of fuel utilization. Particularly in the case where maternal nutrition may become limiting, and thus maternal leptin reduced; fetal- and placental-derived leptin would act on the maternal metabolism to ensure that fuel is preferentially utilized by the fetus, rather than rebuilding maternal stores. This hypothesis is consistent with the observation that leptin concentration is higher in arterial than in venous cord blood and suggests that both fetal- and placental-derived leptin targets maternal metabolism.

To consider this interaction further, we hypothesize that, following parturition, and the accompanying sudden decrease in leptin in the maternal circulation, insulin-mediated fuel storage undergoes a rebound phase, in which maternal fuel stores are rapidly rebuilt. In some women, it may be that this rapid phase of increasing fuel storage is not counter-regulated and thus begins a phase in which adipocyte number increases, predisposing the subject to increased fat-to-lean ratio and in the longer-term, in the case of repeated pregnancies, obesity. These factors are also likely to be intricately linked with lactation as serum leptin concentrations in lactating women are lower post-partum, but somewhat higher than in non-lactating women, further demonstrating a role for leptin in mobilizing fuel reserves.[135]

LEPTIN EXPRESSION IN THE FETUS

The finding that leptin levels are increased in pregnancy[53,124,125,126] has raised the possibility that leptin may play an important role in nutritional signaling between mother and the fetus during pregnancy. The expression of leptin by syncytiotrophoblasts[13,55,56] suggests a role for leptin in nutrient transfer. Hoggard and co-workers[50] studied leptin gene expression and localization of leptin protein in a number of tissues in the 14.5-day-old murine fetus by *in situ* hybridization and immunohistochemistry. High levels of leptin and its receptor expressed in fetal bone and cartilage suggested a role for leptin in bone or cartilage development. Leptin receptor mRNA and leptin receptor protein were also identified in leptomeninges and the choroid plexus of the fetal brain and in lung, kidney, heart and liver.

Schubring and co-workers[136] found that maternal serum leptin concentrations do not correlate with birth weight. In contrast, leptin concentrations in umbilical cord blood correlated positively with birth weight and placental weight in humans.[136] Schubring *et al*[136] demonstrated that leptin concentrations were higher in arterial cord blood than in venous cord blood, leading to the suggestion that leptin synthesis by fetal tissue may be greater than by placental tissue. However, evidence from Linnemann *et al*[134] now suggests that fetal and placentally derived leptin may target maternal metabolism as outlined above. The high levels of expression of both leptin and leptin receptors in the placenta and fetus suggest that leptin plays a key role in fetal development. One possible role is that of a fetal growth factor, ie a signal to the fetus of the maternal energy status. Alternatively, as the concentration of leptin in arterial cord blood is higher than in venous cord blood, leptin may signal to the

placenta the energy status of the fetus, and may have further actions on maternal metabolism, particularly if maternal energy stores become limiting.

LEPTIN AND THE ONSET OF PUBERTY

The weight hypothesis of the development of puberty states that, when body weight reaches a certain level, puberty occurs. If rats are underfed, puberty is delayed, but with access to food rapid weight gain leads to onset of puberty.[137] It has been shown that exogenous leptin accelerates the onset of puberty in normal female mice[124,138] and normal prepubertal female rats.[139] From this evidence it may be speculated that puberty is induced when fat stores reach a certain level, increasing the release of leptin from adipocytes into the bloodstream. Leptin may then act on hypothalamic cells to stimulate release of LHRH, thereby triggering gonadotropin release. The subsequent release of follicle-stimulating hormone (FSH) and LH stimulates gonadal steroid secretion, leading to development of the reproductive tract and induction of puberty.

However, several studies[140,141,142] have demonstrated contrasting data which indicate that the timing of the onset of puberty in male Rhesus monkeys is not triggered by rising circulating leptin concentrations. Given the conflicting reports in the literature,[124,142,143] it is likely that leptin is a factor permissive to the onset of puberty, and that, although some minimal threshold level of leptin is necessary to pubertal development, leptin is more likely not sufficient to initiate puberty. The interactions between leptin and oestrogens and androgens indicate that leptin's role in the onset of puberty is mediated by different mechanisms in males and females and

that further work is required to elucidate these differences. The notion that leptin is a permissive factor is supported by data which show that leptin is also expressed in skeletal muscle tissue.[144,86,82,89] Thus, leptin may act as an indicator of general well-being: that sufficient muscle mass has developed (and is in a phase of anabolic growth) and that a commensurate level of body fat has been deposited, such that it is favourable for puberty (and its consequences, such as pregnancy) to proceed.

LEPTIN AND INSULIN

Insulin is an important regulator of energy homeostasis. It stimulates glucose, free fatty acid and amino acid uptake by tissues and tissue anabolism. It is not surprising that a link between leptin and insulin should exist in the regulation of energy homeostasis.

Leptin appears to act as a permissive factor with respect to its effects on insulin-mediated fuel storage in muscle and in the liver and on insulin-mediated fuel utilization by muscle.

EFFECTS OF INSULIN ON LEPTIN

Current knowledge suggests that insulin plays a chronic role in the regulation of leptin gene expression and production by WAT.

Effects of Hyperinsulinemia

Some studies have shown that hyperinsulinemia increased plasma leptin concentrations and gene expression in WAT in both rodents and humans.[145-153] Recent studies have shown that humans and rodents with insulinoma also have increased

plasma leptin and leptin mRNA and plasma leptin concentrations returned to normal after removal of the insulioma.[154-156] Insulin appears to act directly at the level of the adipocyte by increasing leptin secretion and gene expression, perhaps due to increased glucose transport and metabolism.[158]

Effects of Insulin Deficiency

Streptozotocin-induced insulin deficiency results in reduced circulating concentrations of leptin and mRNA and that suppression was rapidly reversed by treatment with insulin.[159,158]

EFFECTS OF LEPTIN ON INSULIN SECRETION AND TISSUE SENSITIVITY TO INSULIN

In 1996, Kieffer et al102 reported that leptin receptors are expressed in the insulin producing β-cells within the pancreatic islets, suggesting that leptin might influence insulin secretion through a direct action on these cells. This hypothesis was investigated by several others,[160-164] with apparently conflicting results.Thus,the leptin, insulin interaction is highly controversial.

Leptin impaired insulin-mediated stimulation of glucose transport, glycogen synthase activity, lipogenesis, protein kinase A activation and protein synthesis in a dose-dependent manner. Leptin inhibition of insulin-mediated glucose transport appears to be a direct (peripheral) relationship in adipocytes. However it appears to be centrally controlled in muscle tissue .

Other studies have found a direct link between plasma leptin concentration and measures of insulin sensitivity independent of the degree of body fatness.[160,165,166]

High levels of leptin were associated with insulin resistance independent of BMI. In humans, it is still not clear whether the hyperinsulinemia that accompanies insulin resistance is associated with the higher leptin levels, so observed in some obese subjects with non-insulin-dependent diabetes mellitus (NIDDM)[167] or polycystic ovary syndrome (PCOS).[168]

Furthermore, the mechanism underlying the contribution of leptin to insulin secretion as well as the physiological impact of leptin's nutrient stimulation effect remains to be studied in more detail.

Several studies have suggested that glucose is also an important regulator of leptin expression and secretion.[169,170] Kamohara et al[171] have demonstrated that leptin administered into wild-type mice increased glucose turnover and glucose uptake, independent of increase in plasma insulin. However, given that leptin modifies insulin sensitivity of muscle and liver to glucose uptake , probably via CNS control, it appears likely that glucose is regulated and is not a regulator.

LEPTIN AND INSULIN SENSITIVITY OF SKELETAL MUSCLE

It is worthy to note that skeletal muscle accounts for a large proportion of insulin-stimulated glucose uptake and whole body lipid oxidation and is the major contributing tissue to resting metabolic rate. Thus, muscle may be an important tissue for the expression of the juxtaposed interactions of leptin and insulin.

Recently it was reported that leptin directly stimulates fatty-acid oxidation in muscle by activating the 5'-AMP-activated protein kinase, an enzyme that

phosphorylates and subsequently inactivates CoA carboxylase.[172] Thus a direct mechanism for the action of leptin on fatty acid oxidation has now been defined.

EFFECT OF LEPTIN ON GLUCOSE TRANSPORT

Kamohara et al[171] suggested that leptin may acutely increase skeletal muscle glucose uptake by an insulin-independent mechanism. Infusion of murine leptin into the mice led to an increase in glucose turnover and 2-deoxyglucose uptake into skeletal muscle and increased whole-body glucose turnover and glucose oxidation, despite no change in plasma insulin or glucose concentrations.

EFFECT OF LEPTIN ON LIPID METABOLISM

Muscle plays an important role in clearance of serum free fatty acids (FFA) and triacylglycerol (TAG) and in whole-body FFA oxidation. Muoio et al[173] found that leptin increased FFA oxidation in muscle whereas insulin decreased muscle FFA oxidation . One interpretation of these data links the apparent differing responses of muscle and adipose tissue to the interaction between leptin and insulin: obese subjects, having proportionately greater fat mass, may show a direct peripheral effect of leptin suppressing insulin-mediated glucose transport in adipose tissue, while the lipid oxidation effects are mostly manifested in muscle tissue. On the other hand, in lean subjects, having much lower fat mass, most of the effects on turnover are manifested in muscle tissue, and the relativity of the circulating concentrations of leptin and insulin, being lower, would favour lower FFA oxidation in muscle of lean compared to obese subjects. This relationship would be further complicated by the generally increased insulin resistance of obese subjects.

Studies also indicate that leptin attenuates insulin's antioxidative, lipogenic actions on muscle FFA metabolism via a peripheral mechanism, while leptin mediation of insulin-stimulated glucose disposal appears to occur via a central mechanism. Furthermore, as skeletal muscle represents approximately 40% of the total body weight, leptin-stimulated FFA oxidation in skeletal muscle may represent a very important anti-obesity mechanism independent of the central nervous system.

INTERACTION OF LEPTIN WITH INSULIN-MEDIATED HEXOSAMINE SYNTHESIS

Several studies have demonstrated a clear interaction between intracellular hexosamine concentrations (in muscle and in fat) and insulin resistance, in the rodent model, using *in vivo*[175-178,144] and *in vitro*[144,179] methods. This interaction has also been demonstrated *in vitro* in both human myogenic cells[180] and adipocytes.[175] Importantly, in the rodent model, an increase in leptin gene expression was associated with insulin resistance and intracellular glucosamine concentrations.[144,179,181].

The hexosamine biosynthetic pathway is the primary source of the substrates for the glycosylation of (glyco-) proteins,[182] and is hypothesized to be a cellular 'sensor' of energy availability. [175,176,144] However, quantitatively, only 1-3% of incoming glucose ultimately enters the hexosamine pathway.[177,182] An alternative perspective to this proposed model is that, *in vivo*, it is increased leptin which signals excess energy stores and that leptin-mediated damping of insulin actions (particularly in muscle) on both glucose and in this case hexosamines reduces the activity in these storage pathways. In this alternative model, as leptin concentration increases in response to increasing fat mass (muscle has already become loaded with glycogen and hexosamines), the actions of insulin on glucose and hexosamine storage become

increasingly damped. Simultaneously, TAG and thus, fatty acids are preferentially utilized as an energy source. This leads to a brief discussion of leptin interaction with insulin resistance.

LEPTIN AND INSULIN RESISTANCE IN MUSCLE

A vast majority of studies of insulin resistance concentrate upon insulin interactions with glucose, while less attention is paid to insulin-mediated fatty acid uptake, for which mechanisms are now becoming clarified.[183], Although insulin actively increases free fatty acid uptake by muscle,[183,185,185] it clearly inhibits oxidation of free fatty acids, an action which is suppressed by leptin.[186,173,187,188] This partitioning of insulin actions, some of which appear to be synergistic with leptin actions, such as increased uptake of free fatty acids by a direct mechanism and uptake of glucose possibly via a CNS-mediated mechanism (above), and others of which may appear to be antagonistic to leptin actions, such as the effects on fatty acid oxidation [186,173,187,188] are difficult to resolve into a single model. However, it may be stated in broad terms that insulin is anabolic and tends to increase storage of fuels and amino acids, while leptin appears to mobilize TAG and modulate preferential utilization of fatty acids. Simultaneously, leptin inhibits insulin-mediated mechanisms of long-term glucose storage and oxidation.

It is this sub-set of insulin actions upon which many investigations of insulin resistance have focussed and it may be that the typical, many-fold increases in circulating leptin concentrations observed in obesity have profound effects only upon glucose-metabolism-linked peripheral insulin resistance through the mechanisms proposed here. Thus, more thorough investigations of the interaction of leptin and

insulin in regulating both glucose and fatty acid oxidation are of paramount importance in clarifying the progression of obesity and the development of peripheral insulin resistance.

INTERACTION OF LEPTIN WITH OTHER METABOLITES AND HORMONES

Recent studies have shown that free fatty acids (FFA) decrease leptin mRNA levels in adipocytes, suggesting that FFA are involved in the regulation of leptin production in adipocytes. However, regulation of FFA in vivo involves complex mechanisms mediated by a range of hormones including insulin, growth hormone and leptin. Thus, it is likely that these hormones are the drivers of this interaction.

LEPTIN AND GLUCOCORTICOIDS

Adipocyte culture studies have shown that glucocorticoids stimulate leptin gene expression. Glucocorticoid secretion is linked with meal times in normal humans and rodents, and is increased in Cushing's disease or by glucocorticoid administration to normal volunteers. In humans, glucocorticoids stimulate leptin gene expression and secretion independently of effects on food intake, although increases in insulin or lipogenesis associated with food intake may contribute to leptin production.

Mechanisms of glucocorticoid stimulation of plasma leptin is still unknown. Wabitsch and co-workers have postulated that glucocorticoids may influence leptin gene expression directly and independently of their differentiation-promoting effects on adipocytes or they can induce changes in plasma insulin sensitivity. De Vos *et al* have shown similar effects on leptin expression in rats. However, a later study by the

same group indicates that the leptin gene promoter region does not contain a binding site for the glucocorticoid receptor, thus the effect does not rely on the classical molecular mechanism of glucocorticoid receptor action. However, another possible mechanism includes modulation via the CNS, mediated by NPY.

INTERACTION OF LEPTIN WITH THE GROWTH HORMONE AXIS AND OTHER CYTOKINES

Although most of the interactions of leptin with growth hormone are likely to be centrally mediated, interactions of leptin with major components of the growth hormone axis, such as insulin-like growth factor-1 (IGF-1), appear to be more direct .

LEPTIN AND GROWTH HORMONE

Several groups have reported that leptin regulates growth hormone (GH) secretion in humans, rodents, sheep, and pigs. However, when isolated rat adipocytes were incubated with either GH or insulin-like growth factor-1 (IGF-1) alone, there was no effect on leptin secretion.[62]

LEPTIN AND IGF-1

The effect of IGF-1 on the reduction of fat pad mass is most likely due to a suppression of insulin secretion and thus enhancement of fat mobilization and fatty acid (FA) oxidation. As a consequence, adipose tissue leptin mRNA and serum leptin concentrations decrease as well.

LEPTIN AND OTHER CYTOKINES

The influence of cytokines on leptin mRNA expression and circulating concentrations has been investigated in human subjects. Interleukin-1 (IL-1) was found to induce leptin levels directly or indirectly, by increasing the activity of the

hypothalamic/pituitary axis. Mantzoros and co-workers reported a positive and independent association between tumour necrosis factor-α (TNF-α) levels and circulating leptin concentrations, suggesting that TNF-α may directly induce leptin gene expression in humans, as it does in rodents. These studies may implicate a role for leptin in the pathogenesis of cachexia that is accompanied by increased levels of cytokine in advanced stages of AIDS and cancer.

LEPTIN RESISTANCE AND OBESITY

Although leptin is a circulating signal that reduces appetite, in general, obese people have an unusually high circulating concentration of leptin.[186] These people are said to be resistant to the effects of leptin, in much the same way that people with type 2 diabetes are resistant to the effects of insulin. The high sustained concentrations of leptin from the enlarged adipose stores result in leptin desensitization. The pathway of leptin control in obese people might be flawed at some point so the body doesn't adequately receive the satiety feeling subsequent to eating.

AIM OF THE STUDY

AIM OF THE STUDY

- To assess leptin and C-peptide levels in Cord Blood of Infants born to Diabetic Mothers (GDM and type 2 DM mothers)
- To measure the anthropometric measures of the newborn
    - Birth weight
    - Length of babies
    - Ponderal index
    - Head circumference
- To find if there is any correlation between leptin levels and the C-peptide levels
- To find if the leptin levels correlate with the anthropometric measurements of the newborns
- To find the correlation of C-peptide levels with the anthropometric measurements

To assess if there is any gender difference in leptin levels (difference in leptin levels between male and female babies born to women of all three groups)

MATERIALS AND METHODS

MATERIALS AND METHODS

STUDY POPULATION

After getting approval from Ethical Committee of GGH, Chennai, the study was carried out in IOG & Hospital for women and children, Egmore, Chennai.

Cases :

The study sample comprised 40 babies (20M,20 F) born to GDM mothers and 20 babies(9M,11F) born to type2DM mothers. GDM mothers were diagnosed with 100gm glucose tolerance test (ADA Criteria). Both GDM and Type2 DM mothers were on treatment with diet modifications, exercise and insulin.

Controls :

Consists of 30 babies (15M,15F) born to non-diabetic mothers

Inclusion Criteria

Mothers aged between 25-35 years and without any other medical complications of pregnancy

Exclusion Criteria

Mothers with complications like preeclampsia, preterm deliveries, twin pregnancies and other complications during labour were excluded

Other Parameters Considered:

- Gestational age : Calculated with LMP and USG findings in first trimester
- Birth weight and placental weight measured

- Ponderal index of babies measured using birth weight and length of the babies [Weight (g) *100 / Length $(Cm)^3$]
- Head circumference of babies measured

SAMPLE COLLECTION AND PROCESSING

- 10 mL of Cord blood was collected in plain tubes from umbilical vein with precautions to prevent hemolysis
- Sample collection was completed in 1 month
- Serum was separated and stored at -20^0C till analysis
- Cord serum Leptin and C-peptide measured by ELISA

ESTIMATION OF SERUM LEPTIN

Methodology

Enzyme immunoassay method using leptin elisa kit from diagnostic biochem Canada (dbc labs) (Kit No CAN-L-4260)

Principle of the Test

It is a two-step capture or 'sandwich' type assay. It makes use of two highly specific monoclonal antibodies. A monoclonal antibody specific for leptin is immobilized onto the microwell plate and another monoclonal antibody specific for a different epitope of leptin is conjugated to biotin. In the first step leptin present in the samples and standards is bound to the immobilized antibody and to the biotinylated antibody, thus forming a sandwich complex. Excess and unbound biotinylated antibody is removed by a washing step. In the second step, streptavidin-HRP is added which binds specifically to any bound biotinylated antibody. Again unbound

streptavidin-HRP is removed by a washing step. Next the enzymes substrate TMB is added, forming a blue coloured product that is directly proportional to the amount of leptin present. The enzymatic reaction is terminated by the addition of stopping solution, converting the blue colour to a yellow colour. The absorbance is measured on a micotiter plate reader at 450nm. A set of standards is used to plot a standard curve from which the amount of leptin in patient samples and controls can be directly read.

Reagents Provided

1. Anti-Leptin Monoclonal Antibody Coated Microwell Plate –(One 96 well (12x6) monoclonal antibody-coated microwell plate)
2. Monoclonal Anti-Leptin Biotin Conjugate(Monoclonal anti-leptin antibody conjugated to biotin)-10 ml/bottle
3. Streptavidin-HRP Conjugate Concentrate(Streptavidin Conjugated to horseradish peroxidase)- 0.4 mL/bottle
    a. *Preparation*: We diluted 1:50 in assay buffer (e.g. 40µL of concentrate in 2 mL of assay buffer). Since the whole plate was used, we diluted 240 µL of concentrate in 12mL of assay buffer
4. Leptin Calibrators (Six bottles - 0.5 ml / bottle)

Calibrator	Concentration	Volume/Bottle
Calibrator A	0 ng/mL	0.5 mL
Calibrator B	1 ng/mL	0.5 mL
Calibrator C	5 ng/mL	0.5 mL
Calibrator D	10 ng/mL	0.5 mL
Calibrator E	20 ng/mL	0.5 mL
Calibrator F	50 ng/mL	0.5 mL
Calibrator G	100 ng/mL	0.5 mL

5. Control *(*One bottle - 0.5 mL/bottle)
6. Wash Buffer Concentrate *(*One bottle - 50 mL/bottle)
    a. *Preparation* : We diluted 1:10 in distilled water . Since the whole plate was used we diluted 50ml of the wash buffer concentrate in 450 ml of water
7. Assay Buffer (One bottle - 20 mL/bottle)
8. TMB Substrate (One bottle containing tetramethylbenzidine and hydrogen peroxide)- 16 mL/bottle
9. Stopping Solution (One bottle containing 1M sulfuric acid - 6 mL/bottle)

Assay Procedure

1. All reagents were brought to room temperature before use
2. Working solutions of the streptavidin-HRP conjugate and wash buffer were prepared
3. 20µL of each calibrator, control and serum samples were added into correspondingly labelled wells
4. 80µL of the monoclonal anti-leptin-biotin conjugate was added into each well
5. Incubated on a plate shaker (approximately 200 rpm) for 1 hour at room temperature
6. The wells were washed 3 times with prepared wash buffer (300µL for each wash)
7. 100µL of prepared streptavidin-HRP conjugate was added into each well
8. Incubated on a plate shaker (approximately 200 rpm) for 30 minutes at room temperature

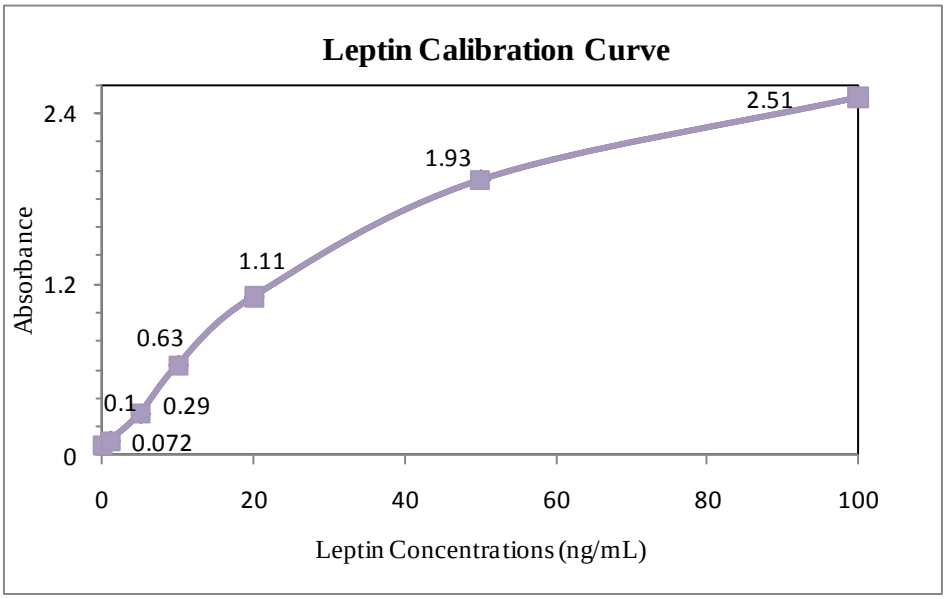
9. The wells were washed again in the same manner as step 6
10. 100µL of TMB substrate was added into each well
11. Incubated on a plate shaker for 10-15 minutes at room temperature
12. 50µL of stopping solution was added into each well
13. The plate was read on a microwell plate reader at 450nm within 20 minutes after addition of the stopping solution

Sensitivity

The limit of detection (LoD) for Leptin is 0.5 ng/mL

Expected Normal Values

Group	Mean (ng/mL)	Range (ng/mL)
Lean Women	7.4	3.7-11.1
Lean Men	3.8	2.0-5.6



ESTIMATION OF SERUM C PEPTIDE

Methodology

Direct solid phase enzyme immunoassay from Diametra for quantitative determination of C-peptide in human serum.

Principle

In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin (S.Avidin) coated on the well and exogenously added biotinylated monoclonal anti-C peptide antibody (Ab). Upon mixing monoclonal biotinylated antibody, the enzyme-labelled antibody and serum containing the native antigen (Ag), reaction results between the native antigen and the antibodies to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$EAb + AgN + BtAb(m) \underset{K\text{-}a}{\overset{Ka}{<->}} EAb - AgN - BtAb(m)$$

BtAb(m) = Biotinylated Monoclonal Antibody (Excess Quantity)

AgN = Native Antigen (Variable Quantity)

EAb = Enzyme labelled Antibody (Excess Quantity)

EAb –AgN – BtAb(m) = Antigen-Antibodies Sandwich Complex

ka = Rate Constant of Association

k-a = Rate Constant of Dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody.

This interaction is illustrated below:

EAb-AgN-BtAb(m) + StreptavidinC.W. => Immobilized Complex

StreptavidinC.W. = Streptavidin immobilized on well

Immobilized complex = sandwich complex bound to the well.

The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce colour. By utilizing several different calibrators of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Test Components

1. C-peptide Standards 6x (1 vial = 2 mL)
2. Conjugate (Anti C-pep-HRP conjugate + Mouse-Anti-C-pep conjugate biotinylated) (1 bottle – 13 ml)
3. Coated Microplate (Microplate coated with streptavidin)
4. TMB-substrate (1 bottle – 12 ml)
5. Stop solution (Sulphuric acid 0.15 mol/L) (1 bottle – 12 ml)
6. Conc. Wash Solution 50x (1 vial) 20 ml (NaCl 9 gr/l; Tween20 1gr/l)

DETECTION LIMIT

This method allows the quantitative determination of C-Peptide from 0.2 to 10.0 ng/ml.

Preparation of the Standard (S0,S1,S2,S3,S4,S5)

The standard has approx. the following concentration:

	S_0	S_1	S_2	S_3	S_4	S_5
ng/ml	0	0.2	1.0	2.0	5.0	10.0

Each standard was reconstituted with 2.0 mL of distilled water.

Preparation of Wash Solution

We diluted the contents of concentrated wash solution 50x to 1000 mL with distilled water in a suitable storage container.

Preparation of the Sample

The samples were stored at temperatures of -20°C .Frozen samples were thawed under tap water.

Assay Procedure

1. All reagents were brought to room temperature
2. 50 µL of each standard prepared (0, 0.2, 1, 2, 5, 10 ng/ml) was added into appropriate wells in the microtiter plate.
3. 50 µL of serum samples were added in the other wells
4. 100 µL of the conjugate was then added to the wells containing standards and serum samples
5. Incubated at room temperature for 2 hours
6. Wells washed with 300 ml of diluted wash solution
7. Washing repeated another two times
8. TMB substrate 100 µL is added to the wells
9. Incubated at room temperature for 15 minutes

10. 100 µL stop solution added to the wells

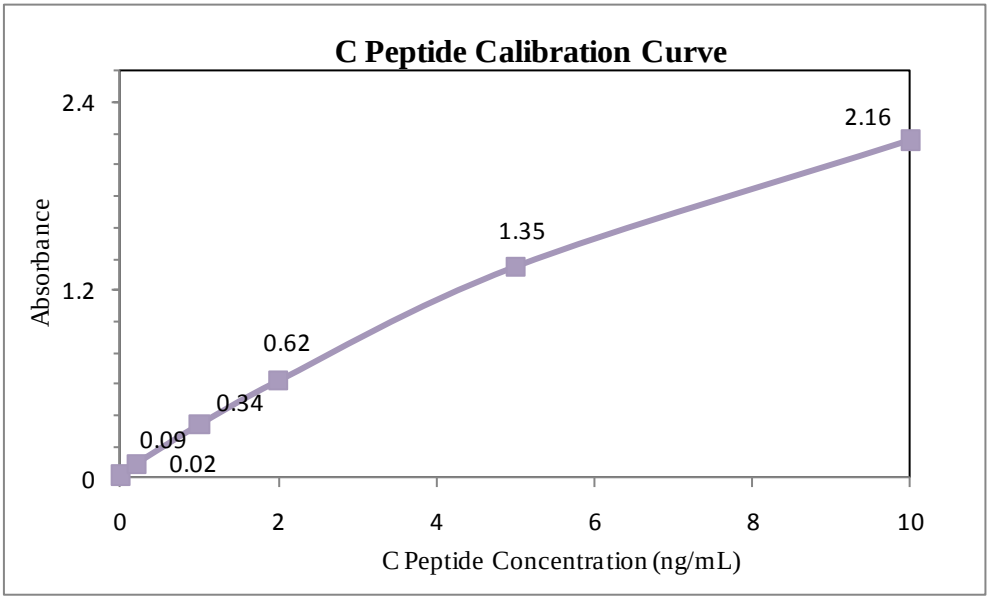
11. The absorbance was read at 450 nm

Reference Value

Adult (Normal) 0.7 – 1.9 ng/mL

Sensitivity

The lowest detectable concentration of C-peptide that can be distinguished from the zero standard is 0.025 ng/mL at the 95 % confidence limit.



RESULTS

Summary of Results

The Various parameters evaluated namely birthweight, head circumference, length of babies, ponderal index, placental weight, gestational age, leptin & C-peptide levels in babies born to GDM, Type2DM and Non diabetic mothers are presented in **Table 1-3** The Mean and Standard deviation for the various parameters evaluated are presented in **Table – 4**

Statistical Analysis

- Statistical Analysis was done by Oneway ANOVA F-test for p-value ($P<0.05$ is considered to be significant)
- Bonferroni t-test was used for comparison of the levels of each parameter in various groups
- Karl pearson's correlation coefficient was used to analyse the correlation between various parameters

Table -4 shows comparison of leptin levels, C-peptide levels, birthweight, placental weight, ponderal index and head circumference among the three groups. Oneway ANOVA is used for the comparison. Leptin and C-peptide levels are higher in cases than in controls. The birthweight, placental weight, ponderal index and head circumference are also higher in cases than in controls.

Table -5 shows the correlation of leptin with C-peptide, birthweight, ponderal index, placental weight, head circumference and gestational age in all the groups. Pearson Correlation is used here. Except for gestational age, all the other parameters show a strong correlation with leptin in all the groups.

Table -6 shows a strong correlation between leptin and C-peptide in all 3 groups.

Table -7 shows a strong correlation of leptin with birthweight, ponderal index and head circumference in all 3 groups.

Table – 8 shows a strong correlation of C-peptide with birthweight, ponderal index and head circumference in the various groups.

Table -9 shows the difference in leptin levels between the male and female babies born to mothers of each group. Student independent t-test is used for this purpose. The difference is significant only in babies of GDM population. The difference is not statistically significant in babies of type 2 DM population and controls probably due to the small sample size of this population.

Table -10 compares the leptin levels in male babies of all 3 groups and in female babies in the various groups. Oneway ANOVA is used here. There is a significant increase in leptin in male babies of GDM and type 2 DM mothers than in controls and a similar increase of leptin in female babies of GDM and type 2 DM mothers compared to controls.

Table -11 shows a comparison of birthweight between male and female babies of mothers of all 3 groups. Student independent t-test is used. There is no statistically significant difference in birthweight between male and female babies in each group.

Table -12 shows a comparison of parity of mothers in all 3 groups. Chi square test is used here. There is no significance.

Table -13 shows a comparison of mode of delivery in all 3 groups. Higher proportion of caesarean section in GDM and DM mothers compared to controls probably due to macrosomia and other complications in them.

SL NO	MOTHER'S AGE (YRS)	PARITY	GESTATIONAL AGE (WEEKS)	MODE OF DELIVERY	SEX OF BABIES	PLACENTAL WEIGHT (GMS)	BIRTH WEIGHT (KG)	LENGTH OF BABIES (CM)	PONDERAL INDEX	HEAD CIRCUMFERENCE (CM)	LEPTIN (ng/mL)	C PEPTIDE (ng/mL)
1	29	2	38	Caesarean	F	445	2.30	46	2.363	33.20	14.91	0.75
2	28	1	38	Caesarean	F	439	2.40	46	2.466	33.10	8.49	1.13
3	30	0	38	Caesarean	M	543	4.30	51	3.242	35.80	71.20	3.20
4	27	0	38	Vaginal	F	468	2.60	47	2.504	34.00	21.65	0.94
5	32	2	38	Caesarean	F	501	2.90	48	2.622	34.20	26.52	1.68
6	33	2	38	Caesarean	F	516	2.90	48	2.622	34.60	34.80	1.42
7	28	0	38	Caesarean	F	511	3.00	49	2.550	34.00	38.10	1.50
8	33	1	39	Caesarean	M	556	4.10	50	3.280	35.90	63.16	3.00
9	30	1	38	Caesarean	F	526	3.00	49	2.550	34.40	35.06	1.46
10	32	0	38	Caesarean	M	537	3.90	50	3.120	35.70	66.50	2.80
11	34	2	38	Vaginal	F	506	3.10	49	2.635	34.50	34.37	1.52
12	30	2	38	Vaginal	F	490	3.10	49	2.720	34.00	38.10	1.75
13	35	1	38	Caesarean	F	502	3.20	50	2.560	34.00	42.10	1.68
14	34	0	38	Caesarean	F	518	3.20	50	2.560	34.60	45.31	1.79
15	35	1	39	Caesarean	M	538	3.70	50	2.960	35.80	59.74	2.80
16	32	1	38	Caesarean	F	535	3.30	49	2.805	35.20	49.61	1.85
17	31	0	39	Caesarean	M	547	3.60	50	2.880	35.50	48.37	2.50
18	33	1	38	Caesarean	M	530	3.30	49	2.805	34.60	37.23	2.60
19	27	0	38	Vaginal	M	536	3.20	49	2.720	34.50	35.50	2.00
20	33	1	38	Vaginal	M	518	3.20	49	2.720	34.80	30.12	1.90
21	27	0	38	Caesarean	F	555	3.60	50	2.880	35.00	55.70	2.80
22	31	1	38	Caesarean	F	550	3.80	50	3.040	35.90	64.92	3.20
23	33	0	39	Caesarean	F	540	3.90	50	3.120	35.60	68.50	3.10
24	28	2	38	Caesarean	M	502	3.10	49	2.635	35.00	30.40	1.80
25	34	2	38	Caesarean	M	508	3.00	49	2.550	35.00	29.60	1.90
26	25	1	38	Caesarean	M	520	3.00	49	2.550	34.80	33.81	1.80
27	34	0	39	Caesarean	F	585	4.10	50	3.280	36.00	70.84	3.20
28	35	0	38	Caesarean	F	570	4.20	50	3.360	35.70	83.70	3.40

Table 1 – MASTER CHART – GESTATIONAL DIABETES MELLITUS

SL NO	MOTHER'S AGE (YRS)	PARITY	GESTATIONAL AGE (WEEKS)	MODE OF DELIVERY	SEX OF BABIES	PLACENTAL WEIGHT (GMS)	BIRTH WEIGHT (KG)	LENGTH OF BABIES (CM)	PONDERAL INDEX	HEAD CIRCUMFERENCE (CM)	LEPTIN (ng/mL)	C PEPTIDE (ng/mL)
29	26	2	39	Caesarean	M	506	2.90	49	2.465	35.00	24.60	1.60
30	31	1	38	Vaginal	M	520	2.90	49	2.465	34.80	30.20	1.50
31	29	2	38	Caesarean	M	504	2.70	49	2.295	34.80	22.44	1.30
32	33	0	38	Vaginal	M	483	2.50	48	2.261	34.90	12.15	1.00
33	28	0	37	Vaginal	M	475	2.50	47	2.408	34.00	19.16	1.20
34	26	1	38	Vaginal	M	452	2.40	47	2.312	34.30	16.03	1.10
35	29	1	37	Vaginal	M	463	2.30	46	2.363	33.80	4.76	0.90
36	34	1	38	Caesarean	F	553	4.20	50	3.360	36.20	74.50	3.30
37	35	2	39	Caesarean	F	560	4.30	50	3.440	35.80	72.10	3.60
38	28	0	37	Vaginal	M	446	2.30	46	2.363	34.00	7.32	0.80
39	26	2	37	Vaginal	M	433	2.20	46	2.260	33.00	5.49	0.80
40	35	1	38	Caesarean	F	560	4.30	50	3.440	35.90	85.23	3.50

Table 1 – MASTER CHART – GESTATIONAL DIABETES MELLITUS

SL NO	MOTHER'S AGE (YRS)	PARITY	GESTATIONAL AGE (WEEKS)	MODE OF DELIVERY	SEX OF BABIES	PLACENTAL WEIGHT (GM)	BIRTH WEIGHT (KG)	LENGTH OF BABIES (CM)	PONDERAL INDEX	HEAD CIRCUMFERENCE (CM)	LEPTIN (ng/mL)	CPEPTIDE (ng/mL)
1	30	0	38	Vaginal	M	465	2.40	46	2.466	34.50	18.75	1.20
2	33	0	38	Caesarean	F	543	4.40	51	3.317	36.30	80.70	3.30
3	31	1	37	Vaginal	M	453	2.50	47	2.408	34.00	9.40	1.33
4	30	1	38	Vaginal	M	464	2.50	47	2.408	34.60	23.60	1.10
5	31	2	38	Vaginal	M	487	2.70	48	2.441	34.70	28.78	1.40
6	33	0	38	Caesarean	F	547	4.30	50	3.440	35.80	76.90	3.30
7	32	2	28	Vaginal	M	505	2.90	48	2.622	35.00	26.40	1.40
8	33	1	38	Caesarean	F	559	4.00	50	3.200	35.60	64.80	3.10
9	34	0	38	Caesarean	F	543	3.60	50	2.880	35.30	58.14	2.90
10	30	0	38	Caesarean	F	555	3.30	49	2.805	35.00	33.31	2.60
11	31	1	38	Caesarean	F	516	3.20	49	2.720	35.00	34.00	2.20
12	31	0	38	Caesarean	F	521	3.10	49	2.635	35.00	30.16	2.20
13	31	0	38	Caesarean	M	532	3.40	49	2.890	35.40	41.50	2.30
14	32	0	38	Caesarean	M	545	3.60	50	2.880	35.70	48.23	2.40
15	29	1	38	Caesarean	F	500	2.90	49	2.465	35.00	26.50	1.90
16	30	1	38	Caesarean	F	480	2.60	48	2.351	34.60	19.37	1.70
17	32	1	37	Caesarean	F	436	2.10	45	2.305	33.40	8.41	1.00
18	33	0	38	Vaginal	F	578	4.50	51	3.392	36.10	83.50	3.20
19	34	0	38	Caesarean	M	548	3.70	50	2.960	35.70	65.40	2.40
20	32	0	38	Caesarean	M	562	3.90	50	3.120	35.70	68.60	2.50

Table 2 – MASTER CHART TYPE2 DIABETES MELLITUS

SL NO	MOTHER'S AGE (YRS)	PARITY	GESTATIONAL AGE (WEEKS)	MODE OF DELIVERY	SEX OF BABIES	PLACENTAL WEIGHT (GM)	BIRTH WEIGHT (KG)	LENGTH OF BABIES (CM)	PONDERAL INDEX	HEAD CIRCUMFERENCE (CM)	LEPTIN (ng/mL)	CPEPTIDE (ng/mL)
1	30	2	40	Vaginal	M	450	2.40	46	2.466	34.00	11.53	1.10
2	29	2	39	Vaginal	M	480	3.00	49	2.550	35.00	20.10	1.30
3	33	1	39	Vaginal	M	436	2.10	46	2.157	33.50	2.43	0.80
4	31	0	40	Caesarean	F	546	3.40	49	2.890	34.20	43.18	1.50
5	28	1	39	Vaginal	F	500	2.60	47	2.504	34.80	18.69	1.00
6	34	0	38	Vaginal	M	432	2.20	46	2.260	33.40	6.27	0.70
7	35	1	39	Vaginal	F	427	2.30	46	2.363	33.60	12.31	0.80
8	29	0	40	Caesarean	M	510	3.10	49	2.635	35.00	26.05	1.20
9	33	0	39	Vaginal	M	440	2.30	46	2.363	33.50	9.32	0.60
10	27	0	40	Caesarean	M	513	3.60	50	2.880	35.70	42.17	1.60
11	25	2	39	Vaginal	F	475	2.60	47	2.504	34.50	20.58	1.10
12	28	1	38	Vaginal	F	470	2.40	46	2.466	33.80	15.49	1.00
13	29	0	38	Vaginal	M	455	2.40	46	2.466	34.00	13.62	1.30
14	33	0	40	Caesarean	F	530	3.60	50	2.880	35.60	48.22	1.50
15	30	1	39	Caesarean	F	513	3.20	48	2.894	35.00	40.94	1.30
16	34	2	38	Vaginal	F	447	2.50	47	2.408	34.00	10.40	0.60
17	28	0	38	Vaginal	M	458	2.50	47	2.408	34.00	14.76	0.80
18	30	0	38	Caesarean	F	469	2.50	47	2.408	34.70	16.74	0.70
19	28	0	38	Vaginal	F	440	2.20	46	2.260	33.30	3.82	0.70
20	31	1	38	Caesarean	M	515	2.90	48	2.622	34.60	23.86	1.20
21	32	0	40	Caesarean	F	520	3.60	49	3.060	35.40	52.79	1.50
22	30	2	40	Caesarean	M	516	3.20	48	2.894	34.60	34.51	1.40
23	34	1	39	Caesarean	M	543	3.30	49	2.805	35.20	31.46	1.50
24	26	0	38	Caesarean	M	465	2.50	47	2.408	34.00	13.20	0.90
25	33	2	38	Vaginal	F	437	2.30	47	2.215	33.40	13.72	0.70
26	29	2	39	Vaginal	M	450	2.30	46	2.363	34.00	5.96	0.80
27	31	1	41	Caesarean	F	546	3.30	49	2.805	35.00	36.12	1.40
28	33	0	40	Vaginal	M	500	3.10	48	2.803	34.70	26.27	1.30
29	30	1	41	Caesarean	F	540	3.70	49	3.145	35.80	50.46	1.60
30	35	1	40	Caesarean	F	551	3.60	50	2.880	35.60	51.00	1.60

Table 3 – MASTER CHART - CONTROLS

RESULTS

Parameters	Group						One way ANOVA	Bonferroni t-test
	GDM		TYPE 2 DM		CONTROL			
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation		
Leptin	40.31	22.71	42.32	24.09	23.87	15.48	F=6.74 P=0.002**	Control Vs GDM, Type2
C peptide	2.00	0.88	2.17	0.76	1.12	0.34	F=17.89 P=0.001***	Control Vs GDM, type2
Birth Weight	3.21	.64	3.28	.71	2.82	.52	F=4.47 P=0.01**	Control Vs GDM, Type 2
Length	48.80	1.40	48.80	1.61	47.60	1.40	F=6.85 P=0.002**	Control Vs GDM, Type2
Ponderal Index	2.738	0.357	2.785	0.363	2.592	0.269	F=2.51 P=0.09	Control Vs GDM, Type2
Head Circumference	34.80	0.84	35.12	0.71	34.41	0.69	F=5.40 P=0.006**	Control Vs Type2
Placental Weight	513.67	38.47	516.95	41.08	485.80	40.42	F=5.36 P=0.006***	Control Vs GDM, Type 2
Gestational Age	38.08	0.53	37.40	2.23	39.10	0.96	F=12.29 P=0.001***	Control Vs GDM, Type2
Mothers Age	30.83	3.08	31.60	1.43	30.60	2.69	F=0.89 P=0.41	-

** significant at P<0.05 ** Highly significant at P<0.01 *** Very High significant at P<0.001*

Table 4 – Comparison of Leptin, C Peptide Levels and Anthropometric Measures among various groups

Parameters	Leptin					
	GDM		TYPE 2 DM		CONTROL	
	Pearsons Correlation	Sig (2-tailed)	Pearsons Correlation	Sig (2-tailed)	Pearsons Correlation	Sig (2-tailed)
C Peptide	0.96	0.001	0.95	0.001	0.96	0.001
Birth Weight	0.98	0.001	0.98	0.001	0.97	0.001
Length	0.85	0.001	0.88	0.001	0.88	0.001
Ponderal Index	0.95	0.001	0.96	0.001	0.96	0.001
Head Circumference	0.94	0.001	0.92	0.001	0.80	0.001
Gestational Age	0.42	0.01	0.47	0.01	0.83	0.001
Placental Weight	0.92	0.001	0.92	0.001	0.90	0.001

Table 5 - Correlation of Leptin with C-peptide and other Anthropometric measures

Pearson Correlation Coefficient

$$r = \frac{N(\Sigma XY) - (\Sigma X)(\Sigma Y)}{\sqrt{[N\Sigma X^2 - (\Sigma X)^2][N\Sigma Y^2 - (\Sigma Y)^2]}}$$

Interpretation for r-value

Pearson correlation coefficient is denoted by “r”

“r” always lies between -1 to +1

0.0 – 0.2 Poor correlation

0.2 - 0.4 Fair correlation

0.4 - 0.6 Moderate correlation

0.6 – 0.8 Substantial correlation

0.8 - 1.0 Strong correlation

Group	Parameter		Leptin	C Peptide
GDM	Leptin	Pearson Correlation	1	.957**
		Sig. (2-tailed)	.	.000
		N	40	40
	C Peptide	Pearson Correlation	0.957**	1
		Sig. (2-tailed)	.000	.
		N	40	40
TYPE2 DM	Leptin	Pearson Correlation	1	0.948**
		Sig. (2-tailed)	.	.000
		N	20	20
	C Peptide	Pearson Correlation	.948**	1
		Sig. (2-tailed)	.000	.
		N	20	20
CONTROL	Leptin	Pearson Correlation	1	.956**
		Sig. (2-tailed)	.	.000
		N	30	30
	C Peptide	Pearson Correlation	.956**	1
		Sig. (2-tailed)	.000	.
		N	30	30

** - Correlation is significant at the 0.01 level (2-tailed)

Table 6 - Correlation of Leptin with C-peptide in all 3 Groups

Group	Parameters	Leptin	
		Correlation	
GDM	Birth Weight	Pearson Correlation	.979(**)
		Sig. (2-tailed)	.000
		N	40
	Length	Pearson Correlation	.851(**)
		Sig. (2-tailed)	.000
		N	40
	Ponderal Index	Pearson Correlation	.951(**)
		Sig. (2-tailed)	.000
		N	40
	Head Circumference	Pearson Correlation	.851(**)
		Sig. (2-tailed)	.000
		N	40
TYPE 2 DM	Birth Weight	Pearson Correlation	.974(**)
		Sig. (2-tailed)	.000
		N	20
	Length	Pearson Correlation	.881(**)
		Sig. (2-tailed)	.000
		N	20
	Ponderal Index	Pearson Correlation	.962(**)
		Sig. (2-tailed)	.000
		N	20
	Head Circumference	Pearson Correlation	.922(**)
		Sig. (2-tailed)	.000
		N	20
Control	Birth Weight	Pearson Correlation	.969(**)
		Sig. (2-tailed)	.000
		N	30
	Length	Pearson Correlation	.888(**)
		Sig. (2-tailed)	.000
		N	30
	Ponderal Index	Pearson Correlation	.956(**)
		Sig. (2-tailed)	.000
		N	30
	Head Circumference	Pearson Correlation	.801(**)
		Sig. (2-tailed)	.000
		N	30

*** Correlation is significant at the 0.01 level (2-tailed)*

Table 7 – Correlation of Leptin with Anthropometric Measures

Group	Parameters	C Peptide	
		Correlation	
GDM	Birth Weight	Pearson Correlation	.976(**)
		Sig. (2-tailed)	.000
		N	40
	Length	Pearson Correlation	.823(**)
		Sig. (2-tailed)	.000
		N	40
	Ponderal Index	Pearson Correlation	.961(**)
		Sig. (2-tailed)	.000
		N	40
	Head Circumference	Pearson Correlation	.871(**)
		Sig. (2-tailed)	.000
		N	40
Type 2 DM	Birth Weight	Pearson Correlation	.956(**)
		Sig. (2-tailed)	.000
		N	20
	Length	Pearson Correlation	.914(**)
		Sig. (2-tailed)	.000
		N	20
	Ponderal Index	Pearson Correlation	.927(**)
		Sig. (2-tailed)	.000
		N	20
	Head Circumference	Pearson Correlation	.868(**)
		Sig. (2-tailed)	.000
		N	20
Control	Birth Weight	Pearson Correlation	.911(**)
		Sig. (2-tailed)	.000
		N	30
	Length	Pearson Correlation	.830(**)
		Sig. (2-tailed)	.000
		N	30
	Ponderal Index	Pearson Correlation	.906(**)
		Sig. (2-tailed)	.000
		N	30
	Head Circumference	Pearson Correlation	.793(**)
		Sig. (2-tailed)	.000
		N	30

** Correlation is significant at the 0.01 level (2-tailed)

Table 8 – Correlation of C-peptide with Anthropometric Measures

	Group	Sex	N	Mean	Std. Deviation	Student independent t-test
LEPTIN	GDM	Male	20	32.3890	20.26697	t=2.32 P=0.03
		Female	20	48.2255	22.72249	
	TYPE 2 DM	Male	9	36.7400	20.63124	t=0.93 P=0.36
		Female	11	46.8900	26.67511	
	Control	Male	15	18.7673	11.63725	t=1.88 P=0.07
		Female	15	28.9640	17.47705	

Table 9 – Comparison of Leptin in Male and Female Babies in each group

Group	Male		Female	
	Mean	Standard Deviation	Mean	Standard Deviation
GDM	32.39	20.27	48.23	22.72
TYPE 2 DM	36.74	20.63	46.89	26.68
Control	18.77	11.64	28.96	17.48
Oneway ANOVA	F=7.43 P=0.001 Control Vs GDM, Type2 GDM Vs Control Type2 Vs Control		F=7.48 P=0.001 Control Vs GDM, Type2 GDM Vs Control Type2 Vs Control	

Table 10 – Comparison of Leptin in Male and Female Babies among all groups

	Group	sex	N	Mean	Std. Deviation	Student independent t-test
Birth Weight	GDM	Male	20	3.055	.6186	t=1.58 P=0.12
		Female	20	3.370	.6392	
	TYPE 2 DM	Male	9	3.067	.5852	t=1.23 P=0.23
		Female	11	3.455	.7815	
	Control	Male	15	2.727	.4667	t=1.01 P=0.31
		Female	15	2.920	.5710	

Table 11 - Birth Weight in Male and Female Babies of all Groups

		Group						Chi-square test
		GDM		TYPE 2 DM		Control		
		n	%	n	%	N	%	
Parity	.00	14	35.0%	11	55.0%	14	46.7%	$\chi 2$=3.49 P=0.48
	1.00	15	37.5%	7	35.0%	9	30.0%	
	2.00	11	27.5%	2	10.0%	7	23.3%	
Table Total		40	100.0%	20	100.0%	30	100.0%	

Table 12 - Comparison of Parity of mothers in all groups

Mode of Delivery	Groups					
	GDM		TYPE 2 DM		Control	
	n	%	n	%	n	%
Caesarean	28	70.0%	14	70.0%	14	46.7%
Vaginal	12	30.0%	6	30.0%	16	53.3%

Table 13 - Comparison of Mode of Delivery in all groups

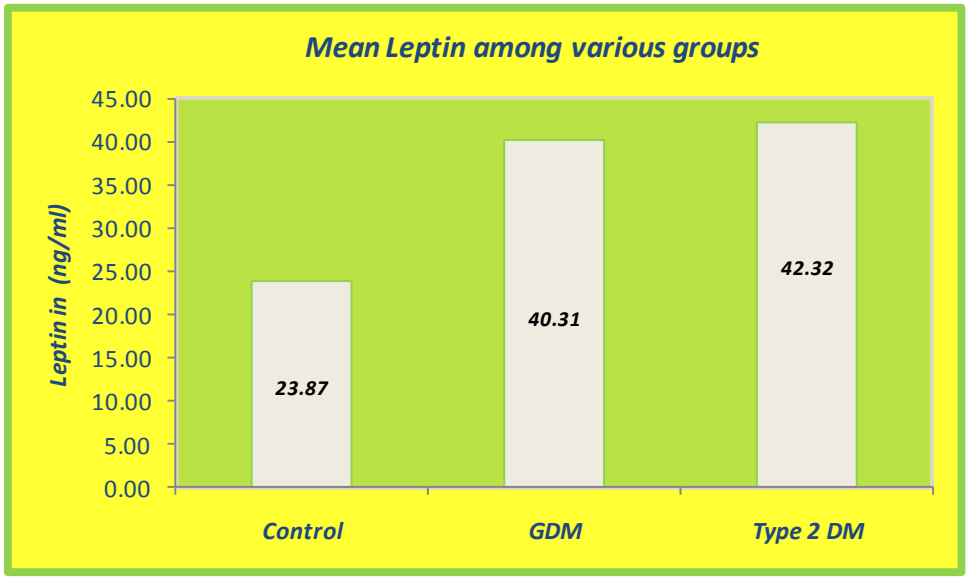


Figure 10 - Leptin among various groups

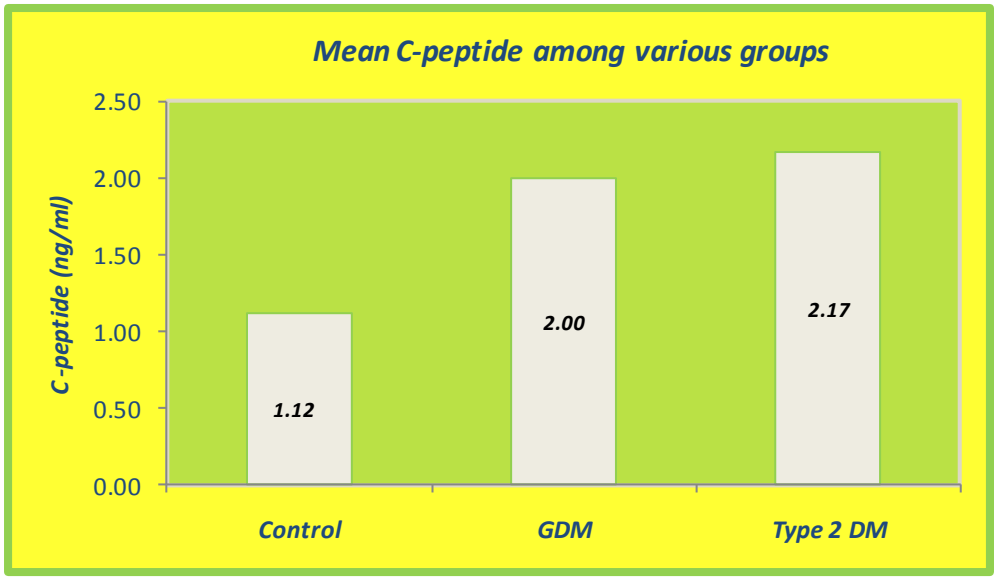


Figure 11- C-peptide among various groups

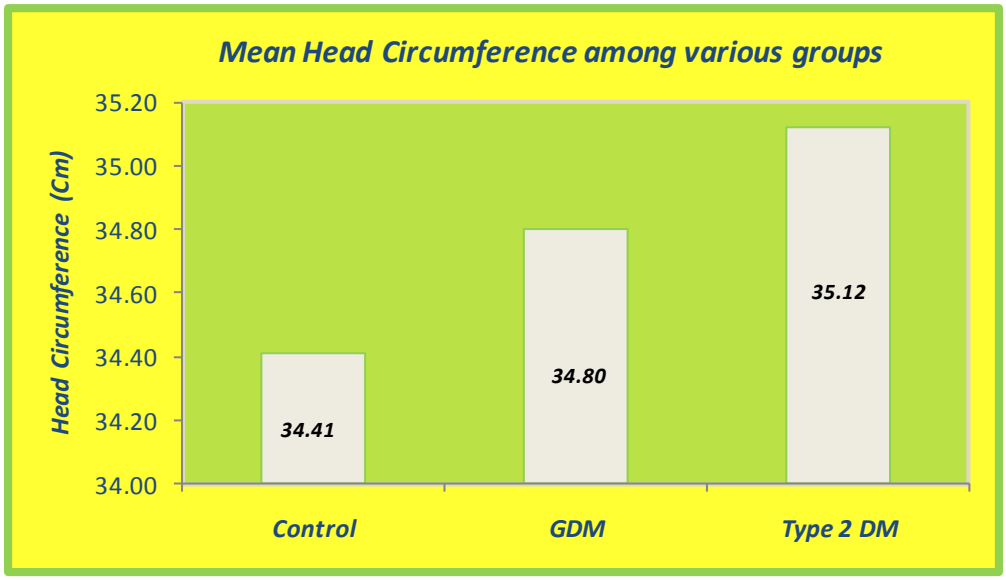


Figure 12- Head Circumference among various groups

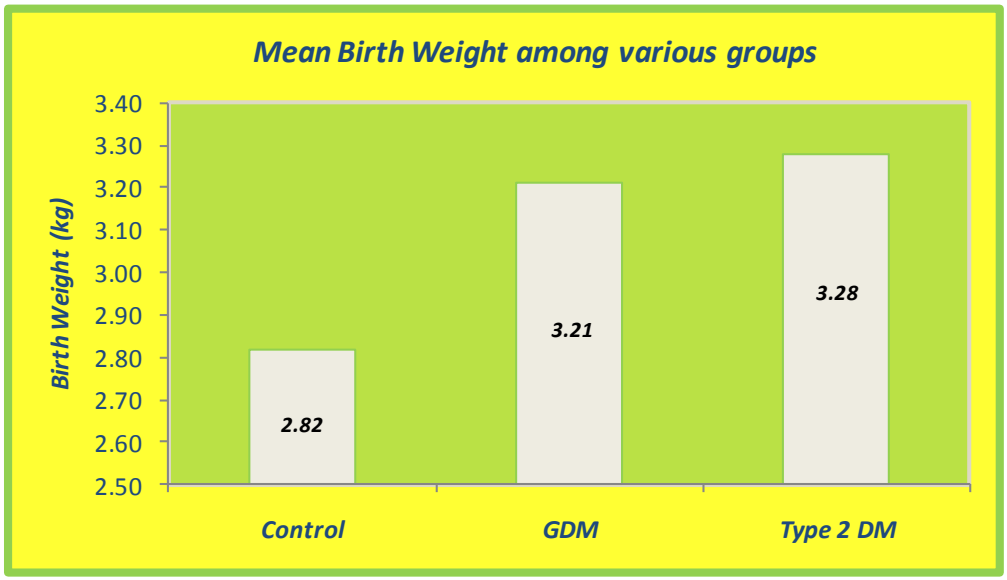


Figure 13- BirthWeight among various groups

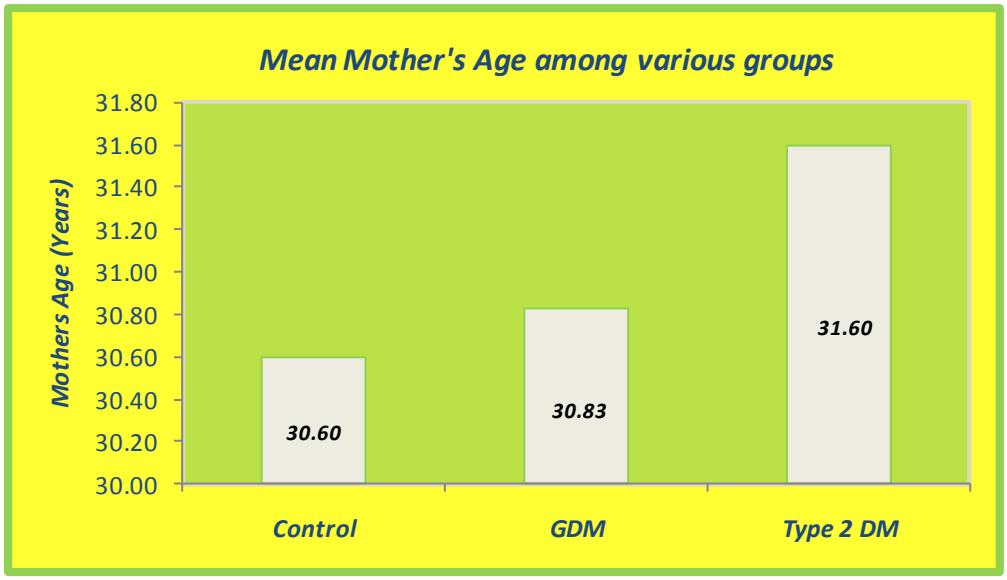


Figure 14- Mother's Age among various groups

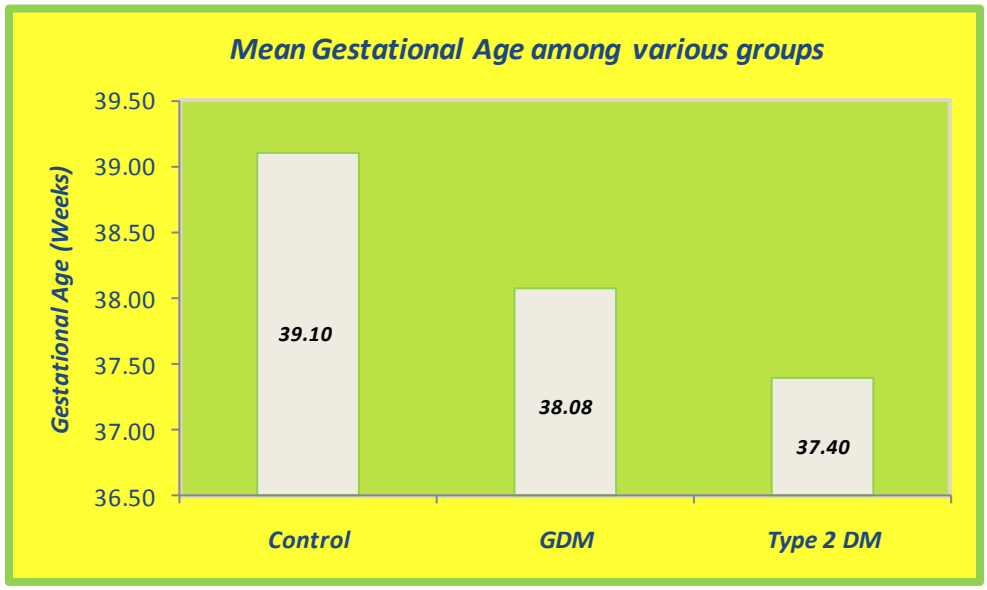


Figure 15- Gestational Age among various groups

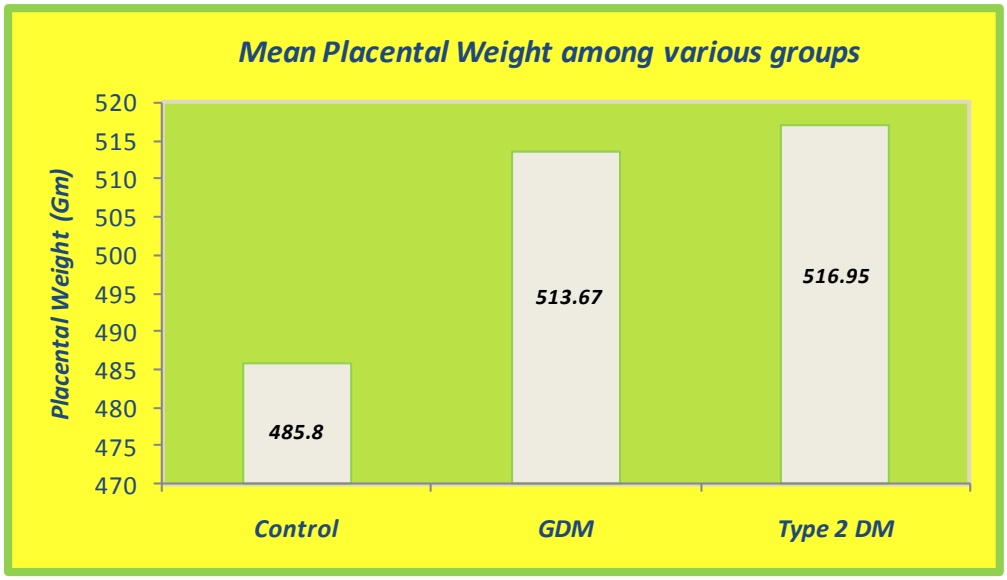


Figure 16- Placental Weight among various groups

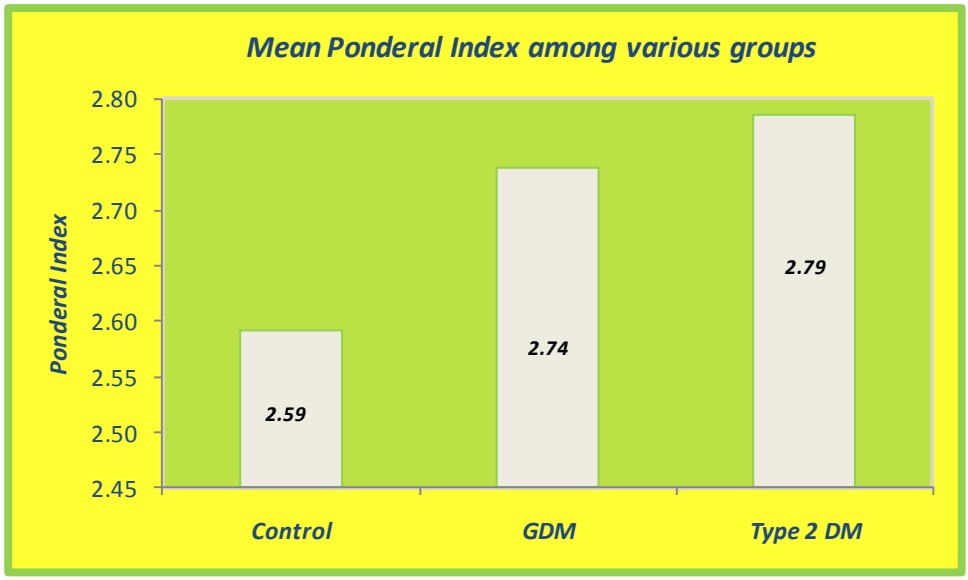


Figure 17 - Ponderal Index among various groups

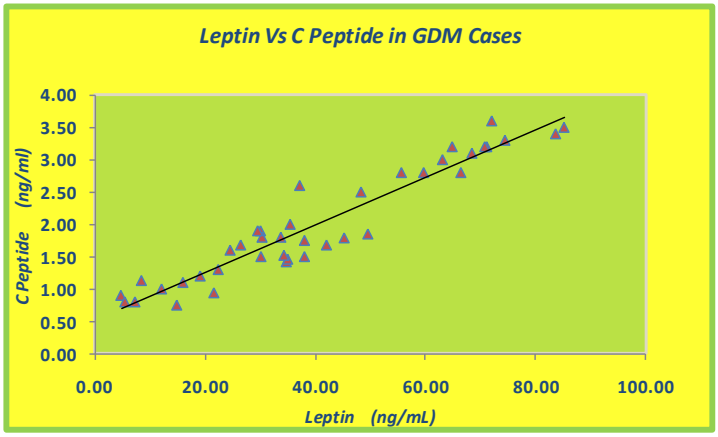


Figure 18- Leptin Vs C-peptide in GDM cases

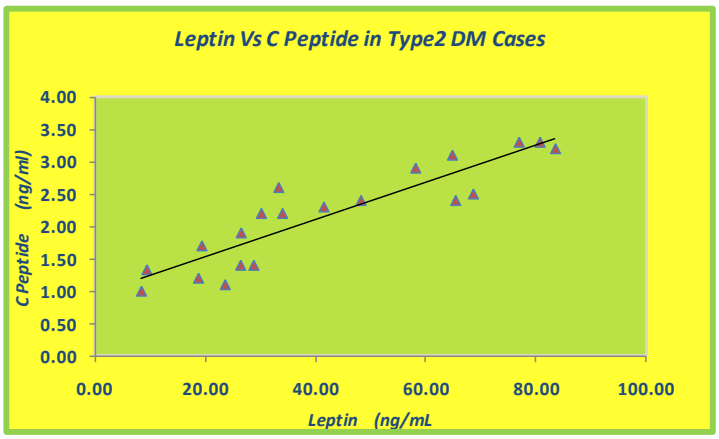


Figure 19- Leptin Vs C-peptide in Type 2 DM

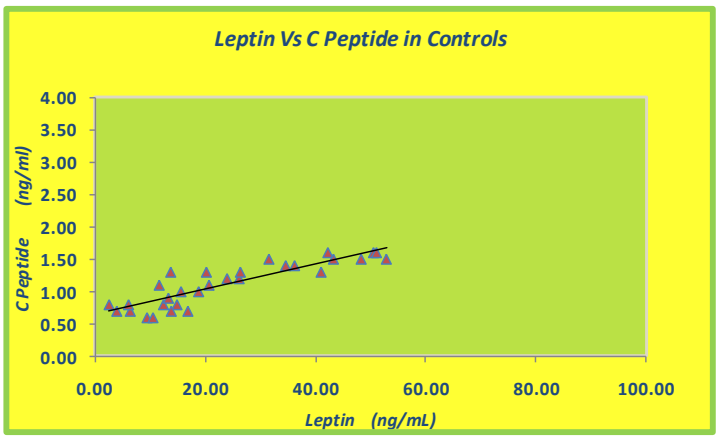


Figure 20- Leptin Vs C-peptide in Controls

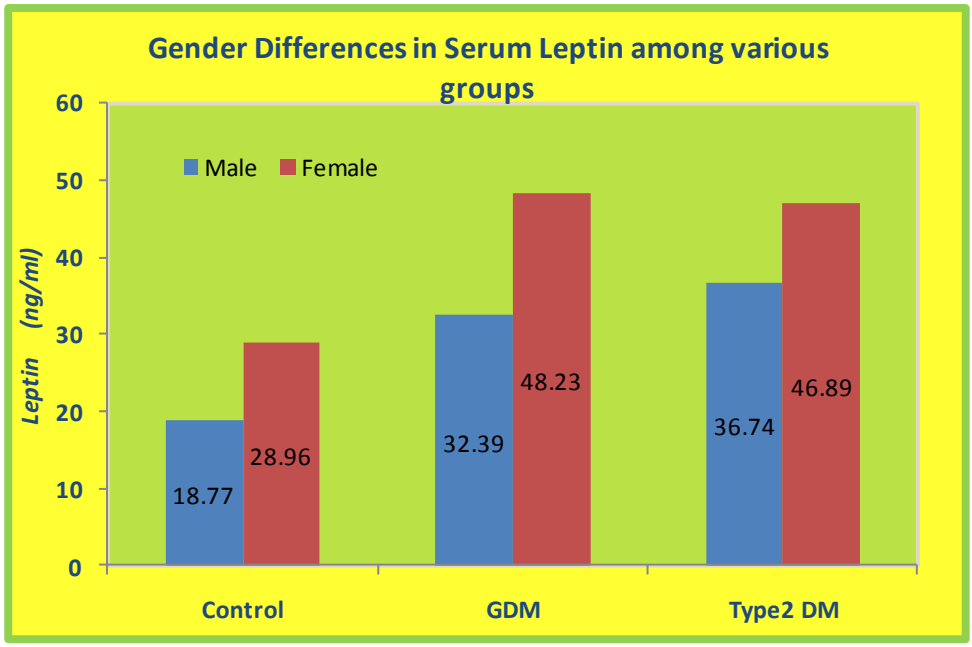


Figure 21- Gender Differences in Serum Leptin among various groups

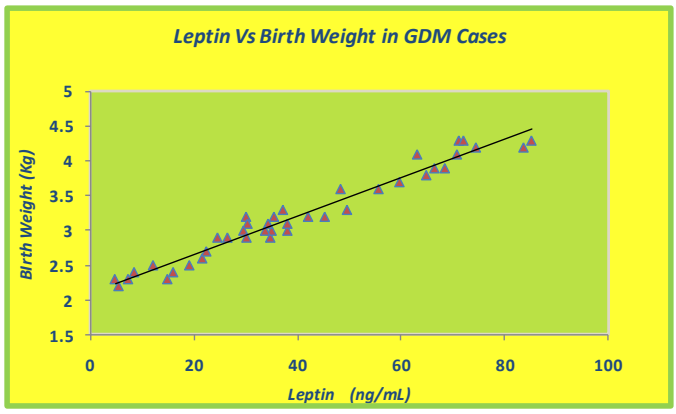


Figure 22 - Leptin Vs Birthweight in GDM Cases

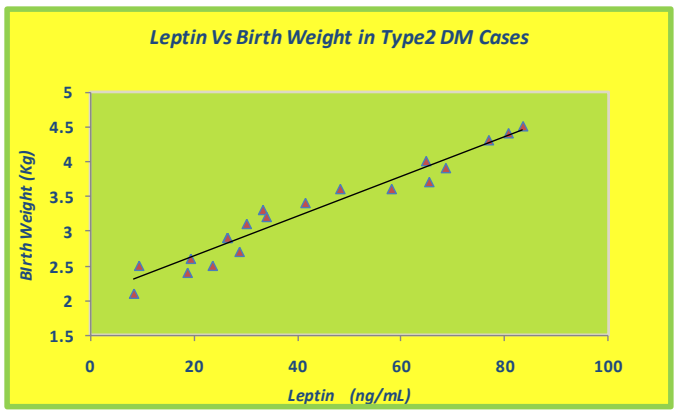


Figure 23 - Leptin Vs Birthweight in Type2 DM Cases

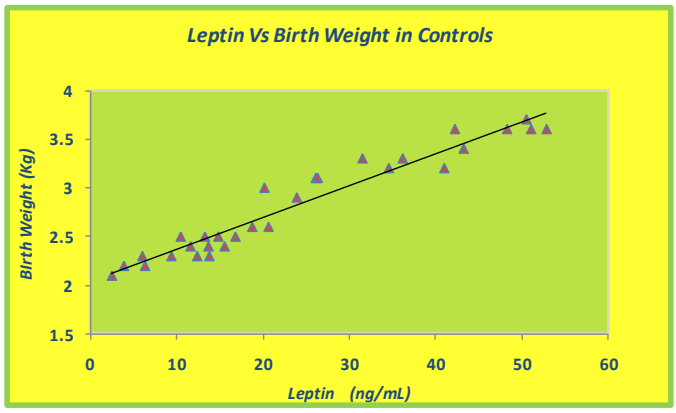


Figure 24 - Leptin Vs Birthweight in Controls

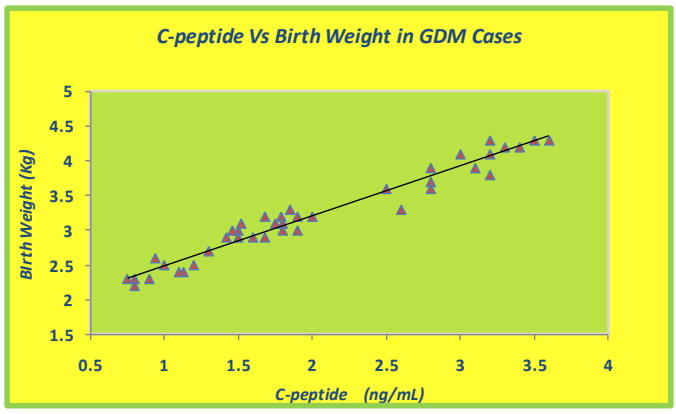


Figure 25 - C-peptide Vs Birthweight in GDM Cases

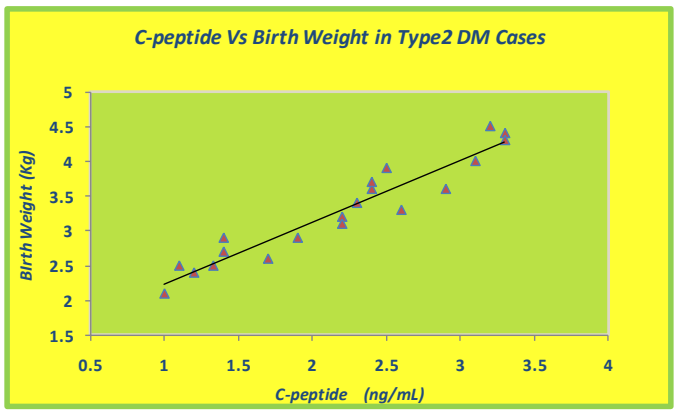


Figure 26 - C-peptide Vs Birthweight in Type2 DM Cases

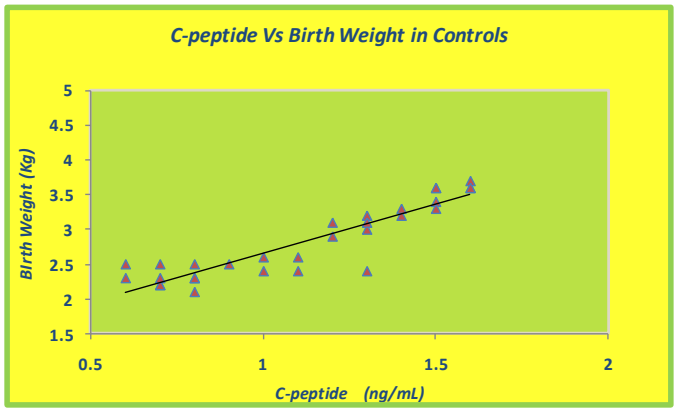


Figure 27 - C-peptide Vs Birthweight in Controls

DISCUSSION

DISCUSSION

Exposure of the fetus to maternal diabetes results in characteristic changes in birth weight, adiposity and fetal insulin production. We have used cord blood insulin and leptin as indicators of exposure to an abnormal intrauterine environment. Notably, insulin and leptin at birth are significantly increased in our population, not only in offspring of mothers with type 2 diabetes, but also in offspring of mothers with GDM as seen in table-4.

Maternal diabetes is clearly associated with an increase in the risk of type 2 diabetes and obesity in the offspring, in keeping with in utero programming of disease[187,188] and forming a "vicious cycle of type 2 diabetes" [189]. More recently, studies[190,191] in hepatocyte nuclear factor-1α mutation carriers (maturity-onset diabetes of the young) have supported a role of the intrauterine environment in modifying the risk of diabetes. Such studies are of interest, as our population is also believed to be at high genetic risk of obesity and type 2 diabetes and therefore likely to be prone to the same programming effects. How important is the finding of raised fetal insulin levels at birth? Importantly, the risk of later adverse metabolic consequences appears to relate to fetal insulin levels[192,193,194]. In this context, our finding of increased concentrations of insulin in cord blood of offspring of mothers with type 2 diabetes and GDM suggests that such a cycle is also potentially present in our population.

The factors that increase fetal leptin levels in macrosomia are not known. In rodents there is very little or no fetal adipose tissue; thus, the macrosomia may be a function of increased placental production, whereas in other animal models, fetal leptin correlates with adipose tissue mass. There is a role for fetal and placental leptin

expression in the regulation of fetal growth, independent of maternal glucose. Placental leptin levels are increased in human diabetic pregnancies[196] and decreased in pregnancies complicated by fetal growth retardation[197]. Leptin's ability to influence fetal growth could have important implications for susceptibility to adult disease.

In our study, we have found a strong correlation between leptin and C –peptide levels in the babies of diabetic mothers, as seen in table-6. A role for leptin in stimulating fetal pancreatic development has been suggested[198,199], which could result in early insulin production and stimulate an increase in fetal growth. Alternatively, fetal hyperinsulinemia could stimulate increased fetal and placental leptin, which, in turn, could contribute to increased fetal growth in tissues expressing the leptin receptor. Studies are currently underway to determine whether maternal leptin administration alters insulin and the b-cell gene expression profile in neonatal mice.

We have also found a significant increase in the birthweight, head circumference and other anthropometric measures in the infants of diabetic mothers and they show a strong correlation with leptin and C-peptide levels as evident from tables 7 and 8.

In a study, Kostolova et al had shown no gender difference in leptin concentration in the offspring of DM mothers. But some studies have suggested the differences in the leptin concentration between sexes.[200,201,202] Various mechanisms have been postulated to explain this difference. The most accepted explanation is the differential adiposity between the genders[203,204,205]. The gender dimorphism in leptin production which is observed in the very early life may also indicate the genetic difference in leptin production. Although we have observed gender difference in leptin levels in our study , between male and female babies born to women in each

group, this difference is significant only in the GDM group and not in the DM group as seen in Table-9. This could be attributed to the reduced sample size in the DM group. However, when we compare the leptin levels between male babies in all 3 groups, there is a significant difference and we observe a similar significant difference when female babies between the 3 groups are compared, as seen in table-10, suggesting that leptin in male babies of diabetic mothers is higher than in non-diabetic mothers. Similarly leptin levels in female babies of diabetic mothers is higher than in non-diabetic mothers.

Table-11 compares the birthweight in male and female babies of each group. There is no significant difference in birthweight between the sexes. So, the increase in leptin levels in female babies of GDM group is independent of an increase in birthweight. Hormonal factors could have contributed to the higher leptin levels.

The importance of GDM as a clinical entity has recently been demonstrated by an Australian randomized trial in which the identification and treatment of GDM significantly reduced the rate of fetal macrosomia and serious adverse perinatal outcome.[198] Thus, the finding that fetal hyperinsulinemia is a common outcome of pregnancy in women with GDM in this population would support the importance of detection and careful monitoring of this group and suggests that similar studies be carried out in other groups.

CONCLUSION

CONCLUSION

- Statistically significant increase in Leptin and C -peptide concentrations in newborns of both type 2 diabetes and GDM mothers is observed
- Similar increase in head circumference, birthweight and other anthropometric measures in the newborns of both type 2 diabetes and GDM mothers is present
- There is a strong correlation between leptin and C-peptide levels in newborns of GDM and type 2 DM mothers
- Leptin levels and C-peptide levels correlate well with the neonatal anthropometric measurements (Birth weight and Head Circumference)
- Leptin concentrations were significantly higher in the female babies in comparison to the male babies in the GDM population. But the difference is not significant in the DM group and controls and this could be attributed to the smaller sample size in these groups

When the mother has type 2 diabetes / gestational diabetes mellitus (GDM), fetal hyperinsulinism is common despite treatment of disease and fetal leptin is also increased. These effects are most pronounced in those women with Type2 DM. This maternal diabetic environment could be a contributing factor for the infants to develop obesity and abnormal glucose tolerance later in life.

SCOPE FOR FURTHER STUDY

SCOPE FOR FURTHER STUDY

- Further studies examining the health of children of mothers with diabetes in this population and whether maternal glycemic control can impact upon this risk, are essential.
- Leptin's ability to influence fetal growth could have important implications for susceptibility to adult disease and will be an important area for future research.
- Regulation of leptin gene expression by androgens and estrogens can be studied.

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BIBLIOGRAPHY

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INSTITUTIONAL ETHICAL COMMITTEE
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L.Dis No.14597/ME5/Ethics Dean/MMC/2010 Dated : 12.01.2010

Title of the work : "Gender differences in serum leptin concentrations from umbilical cord blood of newborn infants born to non diabetic, gestational diabetic and type 2 diabetic mothers".

Principal Investigator : Dr. K. Vani

Designation : PG in MD Biochemistry

Department : Madras Medical College, Chennai-3.

The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 12th January 2010 at 2.p.m in Pharmacology Seminar Hall , Madras Medical College, Chennai -3

The members of the Committee, the Secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the principal investigator.

The Principal investigator and their team are directed to adhere to the guidelines given below:

1. You should get detailed informed consent from the patients/participants and maintain confidentiality.
2. You should carry out the work without detrimental to regular activities as well as without extra expenditure to the Institution or Government.
3. You should inform the IEC in case of changes in study procedure, site investigator investigation or guide or any other changes.
4. You should not deviate form the area of the work for which you applied for ethical clearance.
5. You should inform the IEC immediately, in case of any adverse events or serious adverse reactions.
6. You should abide to the rules and regulation of the institution(s).
7. You should complete the work within the specified period and if any extension of time is required, you should apply for permission again and do the work.
8. You should submit the summary of the work to the ethical committee on completion of the work.
9. You should not claim funds from the Institution while doing the work or on completion.
10. You should understand that the members of IEC have the right to monitor the work with prior intimation.

SECRETARY
IEC,MMC,CHENNAI

CHAIRMAN
IEC,MMC, CHENNAI

DEAN 12/1/10
MADRAS MEDICAL COLLEGE,
CHENNAI -3